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Abstract

The influence of different substrates and the effect of pH regulation during enzymatic hydrolysis of whey protein on glucose-stimulated insulin secretion by BRIN-BD11 pancreatic beta cells were studied. No significant differences ($P \geq 0.05$) were detected in terms of glucose-stimulated insulin secretion by BRIN-BD11 pancreatic beta cells exposed to hydrolysates obtained with two different whey protein substrates WPH1 (5.62 ± 0.42 ng mg^{-1} protein) or WPH2 (5.79 ± 0.35 ng mg^{-1} protein). The whey protein hydrolysate (WPH3) obtained without pH regulation during hydrolysis, had a significantly lower insulinotropic potential (4.10 ± 0.55 ng mg^{-1} protein) in BRIN-BD11 cells than the WPH1 hydrolysate which was manufactured with pH regulation. Fractionation of WPH1 was carried out to enrich bioactive peptides. Comparing the different fractionation techniques studied (solid-phase extraction and semi-preparative reversed phase-high performance liquid chromatography),), the most potent insulinotropic fraction was enriched in free amino acids and contained relatively hydrophilic peptides. This indicates that amino acids and hydrophilic peptides may be involved in the insulinotropic effect of WPH1.

1. Introduction

Consumption of dairy products and particularly low-fat dairy products has been linked with a reduction in the risk of type 2 diabetes (Choi, Willett, Stampfer, Rimm, & Hu, 2005; Tremblay & Gilbert, 2009). The beneficial role of milk proteins, milk-derived peptides and associated amino acids in the regulation of postprandial glycaemia and insulin secretion in normoglycaemic and type 2 diabetic subjects has been demonstrated (Akhavan, Luhovyy, Brown, Cho, & Anderson, 2010; Frid, Nilsson, Holst, & Björck, 2005; Geerts et al., 2011; Manders et al., 2006a; Manders et al., 2006b). *In vitro* studies using pancreatic beta-cell lines or primary islet cells have shown significant acute insulinotropic effects of different amino acid residues (Ala, Leu, Arg and Gln) (Dixon, Nolan, McClenaghan, Flatt, & Newsholme, 2003; Newsholme, Brennan, & Bender, 2006) and incretin hormones (gastro-inhibitory polypeptide-GIP and GIP analogues) (Gault, Flatt, & O'Harte, 2003; Gault, O'Harte, & Flatt, 2003). Ingestion of amino acids (Leu, Arg, Ile, Phe and Ala) or milk-derived peptides with (Geerts et al., 2011; Luhovyy, Akhavan, & Anderson, 2007; Manders et al., 2006a; Manders et al., 2006b) or without carbohydrates (Akhavan et al., 2010; Morifuji et al., 2010; Power, Hallihan, & Jakeman, 2009) has been shown to increase insulin secretion in humans. Intake of hydrolysed milk proteins generally results in higher *in vivo* insulinotropic effects compared to unhydrolysed proteins (Morifuji et al., 2010; Power et al., 2009). However, the mechanisms governing the insulinotropic properties of milk protein hydrolysates are not completely understood. This is illustrated by differences observed in the outcomes of human trials where subjects were administered whey protein hydrolysates. In the study of Akhavan et al. (2010) no effect on post-meal blood glucose was observed following ingestion of a commercial whey protein hydrolysate. In other studies, differences in plasma amino acid composition were seen. Plasma branched chain amino acid (BCAA) levels were increased after ingestion of a whey protein hydrolysate compared to unhydrolysed proteins (Morifuji et al., 2010). In contrast, no difference in plasma BCAA was found

between ingestion of a whey protein hydrolysate and the unhydrolysed protein (Power et al., 2009). It has been suggested that the differences observed between various studies may arise from the protein source and the extent of protein breakdown (Morifuji et al., 2010). To date, most of the hydrolysates which have been used to stimulate insulin secretion *in vivo* have not been characterized in terms of peptide composition thereby making interpretation of results based on peptide bioactive properties problematic.

In a previous study, we developed a whey protein hydrolysate (WPH1) which promoted a strong acute insulin secretion response in the presence of glucose from a clonal rat pancreatic beta cell line (BRIN-BD11) (Gaudel et al., 2013). This hydrolysate also increased insulin secretion *in vivo* when administered by oral gavage for 8 weeks to *ob/ob* mice. This treatment resulted in a restoration of insulin secretion following glucose stimulation of the pancreatic islets isolated from *ob/ob* mice. Treatment with WPH also led to better glucose clearance after a glucose challenge (2 g kg^{-1} body weight) along with an overall decrease in basal plasma insulin in *ob/ob* mice. The peptides in WPH1 were fractionated on the basis of molecular mass differences, however, most of the ultrafiltration fractions generated (permeates and retentates) were insulinotropic when tested *in vitro* with BRIN-BD11 cells (Gaudel et al., 2013).

A significant amount of data exists in relation to the insulinotropic properties of whey protein hydrolysates. However, little information appears in the literature dealing with fractionation of insulinotropic peptides from whey protein hydrolysates (Gaudel et al., 2013). The objective of this study was to determine the influence of two protein substrates and variation in the hydrolysis conditions (with/ without pH control) on the insulinotropic activity of whey protein hydrolysates. Whey protein hydrolyates can be very complex mixtures of free amino acids and peptides, making it difficult to identify the origin of the bioactive components. Therefore, a second objective of this study was to characterise the role of

ultrafiltration, solid-phase extraction (SPE) using a reverse phase polymeric sorbent (Strata-X) and semi-preparative high pressure liquid chromatography (HPLC) in the enrichment of insulinotropic fractions from an insulinotropic whey protein hydrolysate.

2. Materials and methods

2.1. Reagents

Trifluoroacetic acid (TFA), activated carbon (AC), L-glutamine, and D-glucose were obtained from Sigma Aldrich (Dublin, Ireland). Hydrochloric acid (HCl), sodium hydroxide (NaOH), HPLC grade water and acetonitrile (ACN) were from VWR (Dublin, Ireland). Roswell Park Memorial Institute (RPMI) 1640 medium, foetal calf serum, antibiotics (penicillin and streptomycin) and Phosphate Buffer Saline (PBS) were obtained from Invitrogen (Carlsbad, CA, USA). Solid-phase extraction (SPE) Giga Tubes StrataX cartridges (33 µm, 85Å Polymeric RP 1 g, 12 mL tubes) were obtained from Phenomenex (Cheshire, UK). Whey protein substrates WP1 and WP2 were from Carbery Milk Products (Ballineen, Ireland).

2.2. Hydrolysis of whey protein and physicochemical characterisation of the hydrolysates

In order to study the influence of the starting substrate on insulinotropic properties, two different whey protein substrates WP1 (88.3 % (w/w) protein, 1 % (w/w) fat and 2.4 % (w/w) ash) and WP2 (77.9% (w/w) protein, 7.4 % (w/w) fat and 2.8 % (w/w) ash) were hydrolysed. Hydrolysates were generated at pH 7.0, 50°C for 240 min using a pancreatic enzyme preparation as described by Nongonierma & FitzGerald (2012). WP1 and WP2 were used to produce hydrolysates WPH1 and WPH2, respectively. A similar procedure as above was also used to hydrolyse WP1, except that NaOH was not added to control the pH during hydrolysis, this allowed generation of hydrolysate WPH3. The WPH1 hydrolysate was generated as two independent duplicates while WPH2 and WPH3 were generated once. Reproduci-

ble results were obtained during duplicate generation of WPH1 in terms of its peptide composition and insulinotropic properties. For all three hydrolysates, the enzyme was inactivated by heating at 90°C for 20 min, samples were subsequently freeze-dried (FreeZone 18L, Labconco, Kansas City, MO, USA) and stored at -20°C until utilisation.

2.3. Fractionation of WPH1

Different fractionation techniques were used to separate the peptides present in WPH1 as follows:

Membrane processing of WPH1: WPH1 was fractionated using an ultrafiltration (UF) unit (Sartoflow Alpha, Sartorius, Göttingen, Germany) and a Minimate-TFF System (Pall, Port Washington, NY, USA). Fractionation was sequentially carried out using membranes having 5 and 2 kDa (stabilized cellulose) and 0.65 kDa (polyethersulfone) molecular weight cut-off (MWCO) values. The six UF fractions (permeates and retentates) generated were freeze-dried and stored at -20°C until utilisation.

Fractionation by solid-phase extraction: WPH1 was resuspended in HPLC grade water at a concentration of 1 % (w/v) and applied on different adsorbents (Strata-X and AC). The first adsorbent used was a commercial SPE cartridge (Strata-X). The second adsorbent used was AC, which has previously been used for selective binding of hydrophobic peptides (Voirin, Letavernier, & Sebille, 1991). A custom-made SPE cartridge containing AC was assembled as per Nongonierma and FitzGerald (2012). The SPE cartridges were connected to a vacuum (15 mmHg) manifold (Phenomenex) for extraction of different fractions. The procedure followed with both SPE cartridges was a modification of the technique described by Herraiz and Casal (1995). A 40 mL aliquot of WPH1 (1 % (w/v)) was slowly applied at the top of the SPE cartridges to allow adsorption of the peptides. The SPE cartridges were then washed with HPLC grade water (10 mL) to remove any unbound material. Stepwise elution with different solutions (10 mL) of 5 to 50 % (v/v) ACN in water was then used to desorb peptides from the SPE cartridges. At

132 the end of the extraction procedure, the SPE cartridges were washed with 100 % ACN. The fractions
133 obtained were dried in a solvent evaporator (Genevac, EZ-2 Plus, Ipswich, UK).

134 Semi-preparative RP-HPLC: Fractionation of WPH1 was also carried out using semi-preparative RP-
135 HPLC (Waters). WPH1 was resuspended at 12 % (w/v) in HPLC grade water and 500 µL aliquots were
136 injected on a C18 semi-preparative column (250 × 15mm I.D., 10 µm particle size, Phenomenex) at-
137 tached to a C18 guard column (Phenomenex). Mobile phase A was HPLC grade water, and mobile
138 phase B was 100 % (v/v) ACN. The flow rate was set at 6 mL min^{-1} and separation of peptides was car-
139 ried out using the following linear gradient : 0-25 min: 100 % A; 25-80 min: 100-30 % A; 80-85 min:
140 30-0 % A; 85-90 min 0 % A; 90-95 min: 0-100 % A; 95-100 min 100 % A. The absorbance of the elu-
141 ent was monitored at 214 nm. Fractions (56 x 9 mL) were collected using a fraction collector (Waters)
142 during each run. The fractions were pooled into 10 fractions (F1 to F10) collected at 4.5, 10.5, 18.0,
143 24.0, 34.5, 45.0, 54.0, 60.0, 80.0 and 84.0 min, respectively. The semi-preparative procedure was car-
144 ried out in triplicate and individual fractions from the 3 runs in turn were pooled. The different fractions
145 were evaporated to dryness using in a solvent evaporator.

146 *2.4. Reverse-phase high performance liquid chromatography (RP-HPLC) and ultra-performance liquid*
147 *chromatography (RP-UPLC) analysis of the hydrolysates*

148 Unhydrolysed whey proteins (WP1), hydrolysate (WPH1) and ultrafiltration fractions of WPH1 were
149 analysed by RP-HPLC using a Waters (Dublin, Ireland) liquid chromatography system mounted with a
150 250 x 4.6 mm I.D., 5.0 µm Jupiter C18 column coupled to a C18 guard column (4 x 3 mm I.D., Phe-
151 nomenex). Samples were resuspended at 0.8 % (w/v) in 0.1% TFA in HPLC water. Detection of pep-
152 tides and proteins was carried out at 214 nm. Separation was carried out as described in Spellman
153 O'Cuinn & FitzGerald (2009). Whey protein substrates (WP1 and WP2), hydrolysates (WPH1, WPH2

and WPH3) and the SPE fractions were also analysed by reverse phase ultra-performance liquid chromatography (RP-UPLC) using an Acquity UPLC (Waters) as described by Nongonierma and FitzGerald (2012). Samples were resuspended at 0.5 % (w/v) in 0.1% TFA in HPLC water.

2.5. Cell culture and measurement of acute insulin secretion

Acute insulin secretion was measured according to Gaudel et al. (2013). Briefly, BRIN-BD11 cells were seeded at 1.5 x 10^5 in 24-well plates in RPMI-1640 medium to adhere overnight. Medium was removed and cells were washed with PBS before incubation in Krebs-Ringer bicarbonate buffer at pH 7.4 containing 1.1 mM of D-glucose. After 40 min, the buffer was removed and replaced by a Krebs-Ringer bicarbonate buffer at pH 7.4 containing 16.7 mM of D-glucose and an aqueous solution of unhydrolysed whey protein substrates (WP1 and WP2), hydrolysates (WPH1, WPH2 and WPH3) or WPH1 fractions all at 1 mg mL^{-1}. The blank for the assay consisted of Krebs-Ringer bicarbonate buffer at pH 7.4. The positive control was Krebs-Ringer bicarbonate buffer at pH 7.4 enriched with 16.7 mM of D-glucose containing 10 mM alanine. After 20 min incubation, the supernatant was removed and used to quantify insulin secretion using an ultrasensitive insulin enzyme linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden).

2.6. Statistical analysis

Means comparison was carried out with a one way ANOVA followed by a Student Newman-Keuls test using SPSS (version 9, SPSS Inc., Chicago, IL, USA) at a significance level $P < 0.05$.

3. Results

3.1. Influence of the starting substrate and hydrolysis protocol on the physicochemical characteristics and in vitro insulinotropic properties of whey protein hydrolysates

Hydrolysis of WP1 and WP2 was carried out with the same protocol with pH regulation at 7.0 to yield WPH1 and WPH2, respectively. The RP profiles of WP1 and WP2 (Supplementary data Fig. S1A and S1C, respectively) showed that both substrates contained the four major whey proteins (i.e. β-lactoglobulin, α-lactalbumin, bovine serum albumin and caseinomacropeptide). The RP profiles of WPH1 (Supplementary data Fig. S1B) and WPH2 (Supplementary data Fig. S1D) differed with WPH2 containing no residual intact α-lactalbumin and β-lactoglobulin in contrast to WPH1. These differences may arise from the fact that both substrates were injected at the same concentration on a weight basis. The lower protein content of WP2 (77.9 % (w/w)) compared to WP1 (88.3 % (w/w)) yielded lower intensity protein peaks for WP2 compared to WP1. WP1 was hydrolysed using two different protocols. As outlined, WPH1 was generated under controlled pH (7.0) conditions while WPH3 was generated without pH control during hydrolysis. The final pH of WPH3 was 6.2 after 240 min incubation. The RP-UPLC profiles show that WP1 (Supplementary data Fig. S1A) was degraded over the course of hydrolysis, giving a large range of different peptides in WPH1 (Supplementary data Fig. S1B) and WPH3 (Supplementary data Fig. S1E). Differences were evident in the RP profiles of both hydrolysates, lower intensity peptide peaks were present in WPH3 compared to WPH1. In addition, WPH1 only contained trace amounts of the intact proteins (α-lactalbumin, β-lactoglobulin and caseinomacropeptide) whereas WPH3 contained distinct peaks corresponding to unhydrolysed caseinomacropeptide, α-lactalbumin and β-lactoglobulin.

194 The three hydrolysates WPH1 (WP1, pH control), WPH2 (WP2, pH control) and WPH3 (WP1,
195 without pH control) were tested for their insulinotropic properties in the presence of glucose using
196 BRIN-BD11 pancreatic beta cells. The level of acute insulin secretion by the three hydrolysates and the
197 unhydrolysed whey protein substrates is given in Table 1. The insulin secretion values for the hydroly-
198 sates were significantly higher ($P < 0.05$) than for the unhydrolysed whey proteins. No significant dif-
199 ference ($P \geq 0.05$) between WPH1 (5.62 ± 0.42 ng mg^{-1} protein) and WPH2 (5.79 ± 0.35 ng mg^{-1} pro-
200 tein) was seen in terms of insulin secretion. Insulin secretion induced by the addition of WPH3 (4.10 ±
201 0.55 ng mg^{-1} protein) was significantly lower ($P < 0.05$) than that for WPH1 and WPH2.

202 *3.2. Fractionation of WPH1 on the basis of molecular mass and effect on in vitro insulin secretion*

203 UF of WPH1 was carried out using membranes having different MWCO values (5, 2 and 0.65 kDa)
204 allowing generation of six different fractions. The RP-UPLC (Supplementary data Fig. S1A) and the
205 RP-HPLC (Supplementary data Fig. S2A) profiles of WP1 were similar. As expected, under the separa-
206 tion conditions employed, better separation of β-lactoglobulin A and B was obtained in WP1 following
207 RP-UPLC analysis. The RP-HPLC profiles of WP1 (Supplementary data Fig. S2A), WPH1 (Supple-
208 mentary data Fig. S2B) and the 2 kDa permeate of WPH1 (Supplementary data Fig. S2C) were com-
209 pared. The RP-HPLC profile of the 2 kDa permeate differed from that of WPH1, showing a less com-
210 plex peptide profile. The peptides eluting in the hydrophobic region of the RP-HPLC profiles (retention
211 time > 30 min) were present at significantly lower levels in the 2 kDa permeate (Supplementary data
212 Fig. S2C) compared to WPH1 (Supplementary data Fig. S2B). Some peptide peaks present in WPH1
213 were not seen in the 2 kDa permeate, which corresponded to peptides which remained in the retentates
214 at the different stages of the UF procedure (data not shown). As would be expected during enrichment
215 of low molecular mass components during UF processing, some peaks were more abundant in the 2 kDa
216 permeate compared to WPH1. Insulin secretion by BRIN-BD11 cells was stimulated by the 2 kDa per-

meate showing similar ($P \geq 0.05$) secretion to WPH1 (data not shown). Nevertheless, the RP-HPLC profile of the 2 kDa permeate (Supplementary data Fig. S2C) was still relatively complex. Therefore, other fractionation techniques were used in order to enrich for the bioactive peptides in WPH1. This involved SPE with Strata-X (reverse phase polymeric sorbent) and AC cartridges, and semi-preparative RP-HPLC.

3.3. Fractionation of WPH1 via SPE

SPE with Strata-X (SPE X) allowed generation of twelve fractions (Table 2). Mass balance analysis showed 89.1 % (w/w) recovery for this extraction procedure. Insulin secretion by BRIN-BD11 pancreatic beta cells was measured in the presence of glucose plus these fractions and the relative secretion with respect to WPH1 was determined (Table 2). Insulin secretion was significantly higher ($P < 0.05$) than that observed for WPH1 with one fraction (SPE X - unbound). A moderate insulinotropic activity was observed in the fraction SPE X - 40% ACN (Table 2). WPH1 (Supplementary data Fig. S3A), SPE X-unbound (Supplementary data Fig S3B) and SPE X- 40 % ACN (Supplementary data Fig. S3C) showed significantly different RP profiles. As expected, the RP profile of WPH1 showed more peptide peaks than SPE X - unbound or SPE X- 40 % ACN. Peptides in these samples elute according to their relative hydrophobicity. SPE X - unbound contains highly hydrophilic peptides which were not retained on the Strata-X cartridge eluting within the first 15 min of the chromatogram. For SPE X - 40 % ACN, most of the peptide peaks eluted around 20 min, indicating the hydrophobic nature of the peptides present in this fraction.

AC was used in the same fashion as the Strata-X cartridges to fractionate WPH1. Recovery with AC was relatively low (36.9 % (w/w)), showing that a significant amount of WPH1 material remained tightly bound to the AC. Insulin secretion by BRIN-BD11 cells was studied in the presence of the eight frac-

239 tions obtained with the AC cartridge (Table 2). The relative extent of insulin secretion was always lower
240 than that of WPH1 (relative insulin secretion < 1). The highest insulinotropic fraction was SPE AC - 5
241 % ACN. The RP profile of SPE AC - 5 % ACN (Supplementary data Fig. S3D) showed very low inten-
242 sity peaks at 214 nm, which eluted within the first 5 min in agreement with the expected low hydropho-
243 bicity of this fraction.

244 The RP profiles of fractions eluted with 40 % ACN with both adsorbents were compared (SPE X- 40
245 % ACN – Supplementary data Fig. S3C and SPE AC - 40 % ACN – Supplementary data Fig. S3E).
246 Both RP profiles showed very different peptide peaks with most peaks eluting before 20 min with AC in
247 contrast with Strata-X where most peaks eluted after 20 min. This result suggested that AC allowed the
248 production of fractions which were more hydrophilic in nature than Strata-X when eluted with the same
249 ACN concentration.

250 *3.4. Semi-preparative RP-HPLC fractionation of WPH1*

251 WPH1 was injected on a semi-preparative C18 column and ten fractions F1 to F10 were collected.
252 Semi-preparative RP-HPLC allowed collection of a range of hydrophilic and hydrophobic fractions
253 (Fig. 1A). Hydrophilic peptides were eluted with HPLC grade water between 0 and 25 min correspond-
254 ing to fractions F1 to F4. Fractions F5 to F10 were collected during the course of the linear gradient of
255 ACN which was started at 25 min. The ten fractions collected were tested for their insulinotropic activi-
256 ty. Activity in the fractions was expressed relative to the activity obtained with WPH1. F2 which was
257 eluted with water (Fig. 1A) showed the highest relative insulinotropic activity compared to WPH1 (Fig.
258 1B). The insulinotropic activity observed with WPH1 and F2 were not significantly different ($P \geq 0.05$).

4. Discussion

Comparison of the two starting substrates WP1 and WP2 using RP-HPLC analysis showed differences in their susceptibility to hydrolysis. This may be linked to differences in the extent of denaturation of the substrates leading to different susceptibility to enzymatic hydrolysis. The percentage denaturation of the proteins in the starting substrates (WP1 and WP2) was quantified by determining their solubility at pH 4.6 using RP-UPLC (Law & Leaver, 2000). The percentage denaturation for β-lactoglobulin was higher in WP2 (22.0 ± 1.0 % (w/w)) compared to WP1 (11.4 ± 1.1 % (w/w)) whereas denaturation of α-lactalbumin was similar in both substrates (9.9 ± 1.3 and 8.0 ± 0.7 % (w/w) for WP1 and WP2, respectively). The higher denaturation observed in WP2 may explain the lower level of intact whey proteins found in this hydrolysate. Processing parameters can cause protein unfolding resulting in better access by the enzyme to cleavage sites which were previously hidden within the folded protein structure (Hernández-Ledesma, Ramos, Recio, & Amigo, 2006). However, while there were differences in the RP-HPLC profiles of WPH1 and WPH2, the level of insulin secretion by BRIN-BD11 cells treated with these hydrolysates was not significantly different. In a previous study, we demonstrated that the insulinotropic properties of WPH1 when applied to BRIN BD11 pancreatic beta cells translated to an *in vivo* response with *ob/ob* mice (Gaudel et al., 2013).

The influence of the hydrolysis process was investigated in order to determine differences between WP1 hydrolysates generated with pH control at pH 7.0 (WPH1) and without pH control (WPH3). A lower protein breakdown was seen in the RP-HPLC profile of WPH3 compared with WPH1 (Supplementary data Fig. S1B and E). Alteration of pH affects the hydrolytic activity of enzymes thereby affecting protein breakdown. A lower level of insulin secretion was obtained with WPH3 compared to WPH1 (Table 1), suggesting that bioactive amino acids or peptides were released to a lower extent without pH control during hydrolysis. The influence of hydrolysis pH on the bioactive properties of

milk protein hydrolysates has previously been reported in the literature. Van der Ven, Gruppen, de Bont & Voragen (2002) studied the influence of different processing parameters, including hydrolysis pH, during whey protein hydrolysis on angiotensin converting enzyme inhibitory (ACE) activity. They reported an optimum value for hydrolysis pH (between 8 and 9) yielding a minimum IC_{50} for ACE inhibition.

Free amino acid analysis of the different hydrolysates was carried out as per Mounier, Rea, O'Connor, Fitzgerald and Cogan (2007) showing higher levels of total free amino acids in WPH2 (12.7 % (w/w)) and WPH1 (13.8 % (w/w)) compared to WPH3 (8.9 % (w/w)). Little difference in terms of free BCAA was seen between the 3 hydrolysates. However, the levels of Arg and Phe, which have been associated in the literature with an insulinotropic activity (Newsholme et al., 2006), were found in higher amounts in WPH1 and WPH2 (between 1.6 and 2.1 times more) compared to WHP3 (data not shown). It is anticipated that free amino acids and dipeptides would be bioavailable (Foltz et al., 2007; Morifuji et al., 2010) and therefore may reach pancreatic beta cells *in vivo*. It is interesting to note that the free amino acid level in WPH3 was decreased by 36 % compared to WPH1, which is of the same order of the decrease in insulin secretion (22 %) between WPH3 and WPH1. In this specific case, the level of free amino acid may be the main determinant for the insulinotropic properties of WPH3. Addition of base or acid to regulate pH during hydrolysis will result in modifications in the ionic content of hydrolysates (Aspmo, Horn, & Eijsink, 2005). Efforts are currently being made globally to reduce sodium in foods in order to help minimise hypertension in the population (Campbell, Johnson, & Campbell, 2012). Interestingly, in this regard, a significant level of bioactivity could be retained with WPH3 while reducing the sodium content by more than 70 % (w/w) compared to that in WPH1 (Table 1).

The WPH1 hydrolysate was fractionated in order to better characterise the origin of the bioactive components within this sample. The recovery (89.1 % (w/w)) for WPH1 peptides eluted from the Strata-

X SPE cartridge was of the same order as in the study of Herraiz and Casal (1995) and Nongonierma et al. (2013). The Strata-X- unbound fraction contained numerous peaks showing that several peptides had no affinity for the sorbent (Supplementary data Fig. S3B). Adsorption of peptides depends on their affinity for the sorbent. Therefore, peptides in the unbound fraction can be considered to be relatively hydrophilic while peptides eluting with higher ACN concentrations are relatively hydrophobic. WPH1 was composed of peptides having a wide range of hydrophobicities, which is consistent with the fact that different peptide peaks were eluted with increasing ACN concentration in the washing steps (Supplementary data Fig. S3). Peptide recovery from AC SPE was relatively low (36.9 % (w/w)) compared to the recovery obtained using Strata-X SPE (89.1 % (w/w)). This value was of the same order as the recovery found with a lactoferrin hydrolysate (28.0 % (w/w)) (Nongonierma & FitzGerald, 2012). Irreversible adsorption of milk peptides on SPE adsorbents has been described previously (Herraiz & Casal, 1995; Voirin et al., 1991). Differences in peptide binding to AC and Strata-X were seen, with binding to AC involving stronger hydrophobic interactions than binding to Strata-X.

The unbound fraction obtained by Strata-X SPE was the only fraction giving a significantly higher ($P < 0.05$) insulin response than WPH1 (Table 2). It can be seen that the most potent fractions (SPE X - unbound, SPE AC - 5 % ACN and F2), obtained herein using the three fractionation techniques were relatively hydrophilic (Table 2 and Fig. 1A). Interestingly, analysis of the free amino acids present in the unbound material eluting from the Strata-X SPE - and in the 2 kDa permeate of WPH1 showed a significant enrichment in free amino acids, including BCAA, present in these fractions (Fig. 2). BCAA have been associated with insulinotropic effects (Dixon et al., 2003Dixon et al., 2003; Geerts et al., 2011; Luhovyy et al., 2007; Manders et al., 2006a; Manders et al., 2006b; Newsholme et al., 2006) which may be responsible for the insulin secretion observed for these fractions. Twice the amount of free amino acids and BCAA's were found in the Strata-X SPE and in the 2 kDa permeate compared to

WPH1. It would have been anticipated that the insulin secretion observed with these fractions would also double. However, there was only a modest increase in insulin secretion with both fractions. This suggests that free amino acids are not the only components of WPH1 which are responsible for the bioactivity and that some peptides may also influence the insulinotropic properties of WPH1. This is further reinforced by the fact that other hydrophobic fractions were also able to stimulate insulin release from BRIN-BD11 cells (Table 2).

A possible explanation for the apparent inability to increase insulin secretion in comparison to the complete hydrolysate (WPH1) when using various fractions obtained by different fractionation strategies may have been that BRIN-BD11 cells require specific mixtures of peptide combinations to promote insulin secretion. Overall our results herein suggest that the bioactivity observed within the WPH1 hydrolysate may arise from a mixture of free amino acids and peptides having a range of hydrophilic/hydrophobic and molecular mass properties.

Conclusion

The role of free amino acids and bioactive peptides released during whey protein enzymatic hydrolysis on *in vitro* insulin secretion by pancreatic beta cells has been studied. Protein breakdown into peptides and free amino acids is a prerequisite for the bioactive properties of the hydrolysate to be seen. The overall composition of the hydrolysate including its sodium, free amino acid, peptide and residual intact protein content may be modified by altering the hydrolysis conditions, e.g., by including, or excluding a pH adjustment step. This in turn was shown to directly affect the insulinotropic properties of the hydrolysate.

All three fractionation procedures employed to enrich/isolate bioactive components showed that the most potent insulinotropic fractions obtained were hydrophilic. It was shown that these fractions were enriched in free amino acids and also contained hydrophilic peptides, which may both be responsible for the insulinotropic activity observed. However, other peptides with hydrophobic characteristics may also contribute to the insulinotropic properties of the whey protein hydrolysate. The results herein are of significance to our understanding of the characteristics of insulinotropic amino acids and peptides within whey protein hydrolysates.

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Table 1 Insulin secretion by BRIN-BD11 cells treated with unhydrolysed and hydrolysed whey protein substrates.

Sample[1]	Insulin secretion (ng mg^{-1} protein)[2]
WP1	0.90 ± 0.18^{a}
WPH1	5.62 ± 0.42^{b}
WP2	0.90 ± 0.23^{a}
WPH2	5.79 ± 0.35^{b}
WPH3	4.10 ± 0.55^{c}

[1] WP1 and WP2: unhydrolysed whey proteins, WPH1: WP1-derived hydrolysate manufactured with pH regulation at 7.0, WPH2: WP2-derived hydrolysate manufactured with pH regulation at 7.0 and WPH3: WP1-derived hydrolysate manufactured without pH regulation

[2] Insulin secretion values are expressed in ng insulin released per mg of protein in the pancreatic BRIN-BD11 cells. Average values ± SD of three replicates. Values with different superscript letters are significantly different ($P < 0.05$).

Table 2 Relative amount of insulin secreted by BRIN-BD11 cells treated with whey protein-derived hydrolysate (WPH1) fractions obtained on elution from solid-phase extraction Strata-X (SPE X) and activated carbon (SPE AC) with different acetonitrile (ACN) concentrations.

Sample[1]	**Relative insulin secretion**[2]	
	SPE X	**SPE AC**
WPH1	1.06 ± 0.13^{b}	1.00 ± 0.16^{a}
unbound	1.20 ± 0.14^{a}	$0.46 \pm 0.03^{b,c,d}$
5 % ACN	0.21 ± 0.03^{d}	0.70 ± 0.08^{b}
10 % ACN	0.19 ± 0.01^{e}	$0.47 \pm 0.09^{b,c,d}$
15 % ACN	0.28 ± 0.07^{e}	-
20 % ACN	0.27 ± 0.05^{e}	$0.29 \pm 0.00^{c,d}$
25 % ACN	0.26 ± 0.07^{e}	-
30 % ACN	0.19 ± 0.04^{e}	0.25 ± 0.04^{d}
35 % ACN	0.25 ± 0.06^{e}	-
40 % ACN	0.71 ± 0.09^{c}	$0.39 \pm 0.14^{c,d}$
45 % ACN	0.56 ± 0.07^{d}	-
50 % ACN	0.20 ± 0.07^{e}	$0.44 \pm 0.16^{b,c,d}$
100 % ACN	$0.66 \pm 0.06^{c,d}$	0.23 ± 0.05^{d}

[1]SPE X : fractions obtained on elution from solid-phase extraction with Strata-X, SPE AC: fractions obtained on elution from solid-phase extraction with activated carbon with different acetonitrile (ACN) concentrations

[2]Insulin secretion in BRIN-BD11 pancreatic beta cells relative to the secretion observed with WPH1. Average values ± SD of three replicates. In the same column, values with different superscript letters

were significantly different ($P < 0.05$).. The absolute value of insulin secretion obtained for WPH1 was of 5.51 ± 0.69 ng mg^{-1} protein.

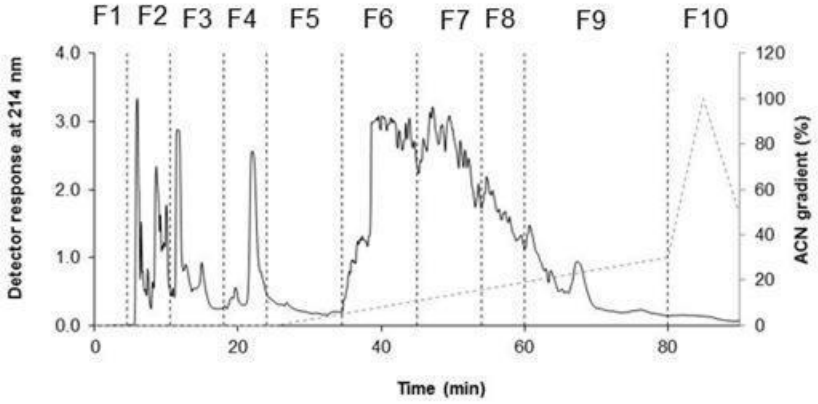
Figure captions

Fig. 1. Reverse-phase high pressure liquid chromatography (RP-HPLC) profile during the semi-preparative separation of whey protein hydrolysate WPH1 (a) and relative insulin secretion in BRIN-BD11 pancreatic beta cells observed with the semi-preparative RP-HPLC fractions of WPH1 (b). Values represent mean insulin secretion relative to the secretion observed withWPH1 ± SD of three replicates, values with different superscript letters are significantly different ($P < 0.05$). The absolute value of insulin secretion obtained for WPH1 was of 2.52 ± 0.40 ng mg^{-1} protein.

Fig. 2. Relative free amino acids amounts in the whey protein hydrolysate (WPH1) fraction unbound to the Strata X solid phase extraction cartridge (SPE X unbound) and its 2 kDa permeate compared to WPH1. Values represent mean free amino acid concentration relative to the whey protein hydrolysate WPH1 ± SD of three replicates. □ : SPE X unbound and ■: 2 kDa permeate.

479

A



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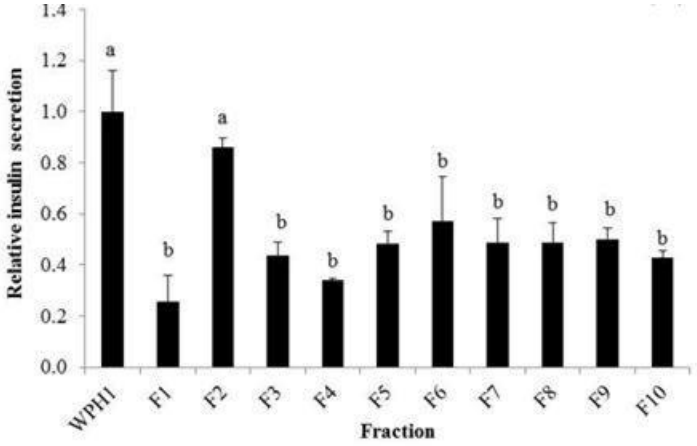

480

481

Fig. 1

482

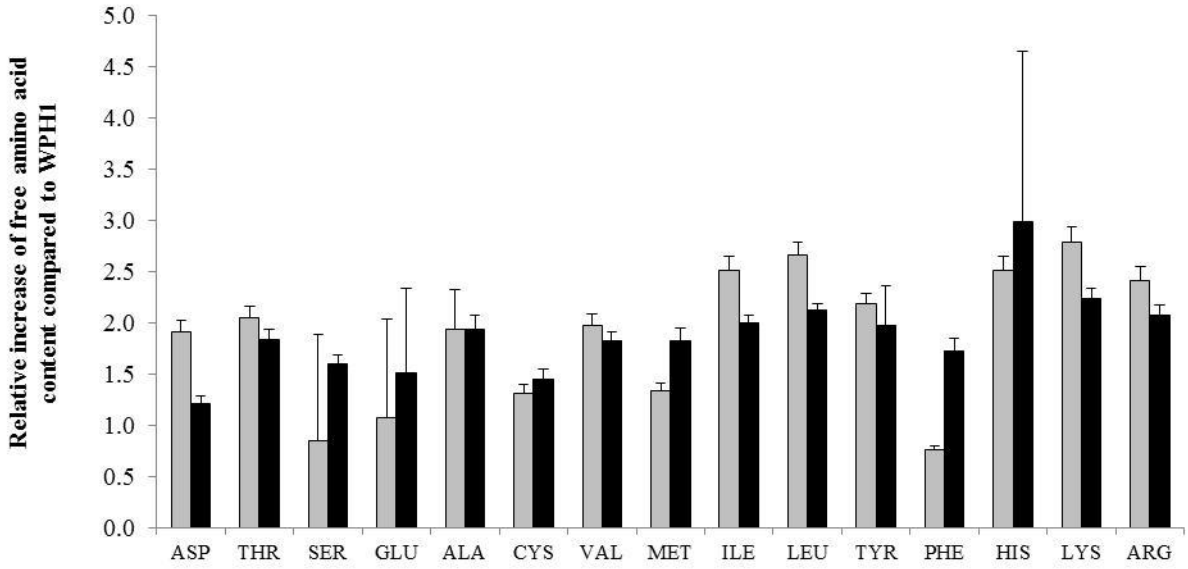
Relative increase of free amino acid content compared to WPH1
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ASP
THR
SER
GLU
ALA
CYS
VAL
MET
ILE
LEU
TYR
PHE
HIS
LYS
ARG

Fig. 2

Supplementary data

Insulinotropic properties of whey protein hydrolysates and impact of peptide fractionation on insulinotropic response

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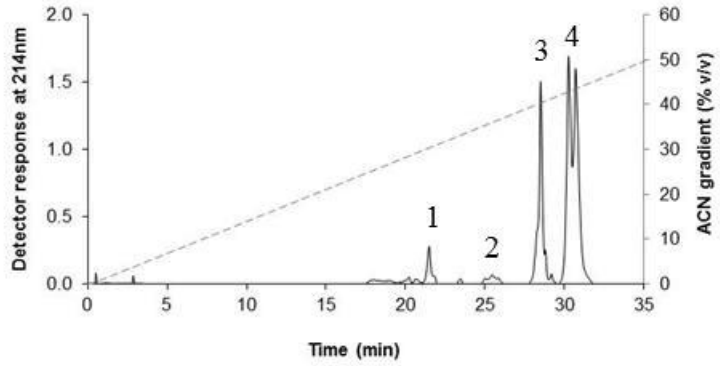
A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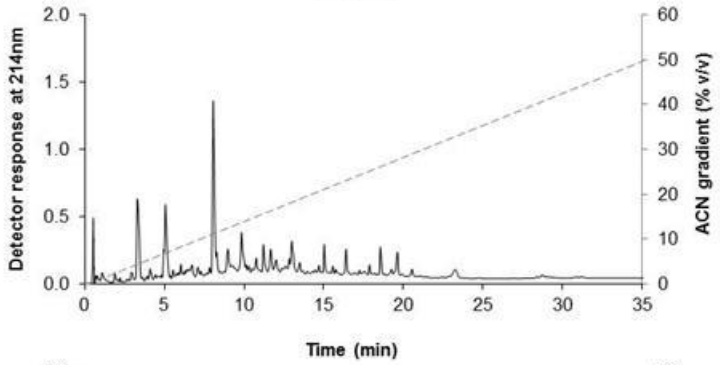
ACN gradient (% v/v)
Time (min)
1
2
3
4

B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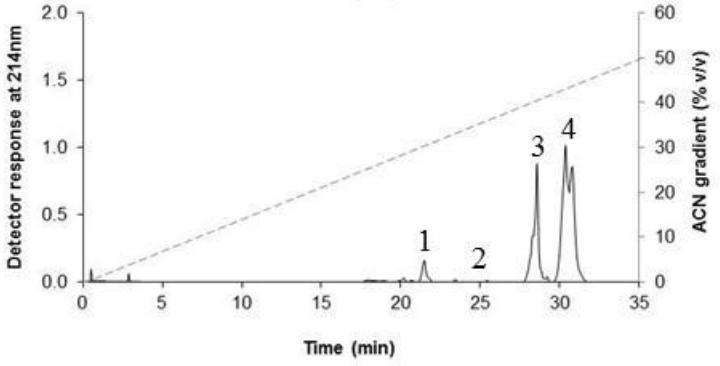
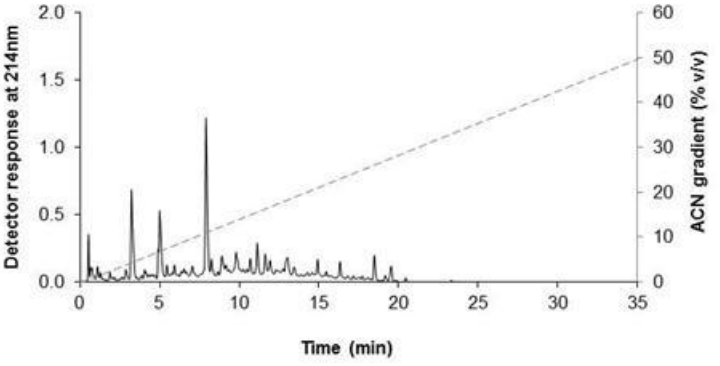
ACN gradient (% v/v)
Time (min)

C


Detector response at 214nm
ACN gradient (% v/v)
Time (min)
1
2
3
4

D


Detector response at 214nm
ACN gradient (% v/v)
Time (min)

E

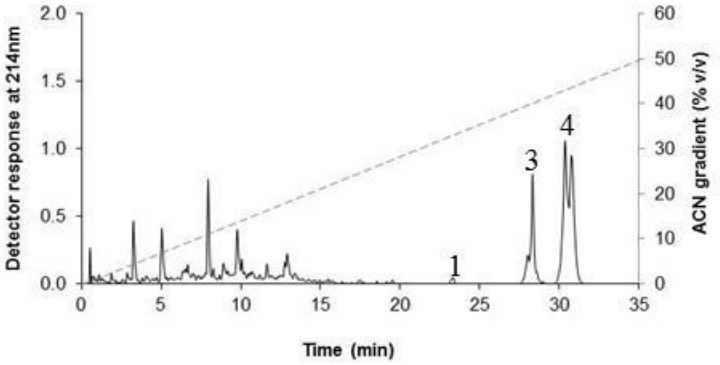

Detector response at 214nm
ACN gradient (% v/v)
Time (min)
1
3
4

Fig. S1. Reverse-phase ultra-performance liquid chromatography (UPLC) profiles of unhydrolysed whey proteins and whey protein hydrolysates. (a) WP1 (b) WPH1 (c) WP2 (d) WPH2 (e) WPH3. 1-caseinomacropeptide, 2-bovine serum albumin, 3- α-lactalbumin, 4- β-lactoglobulin variants A and B.

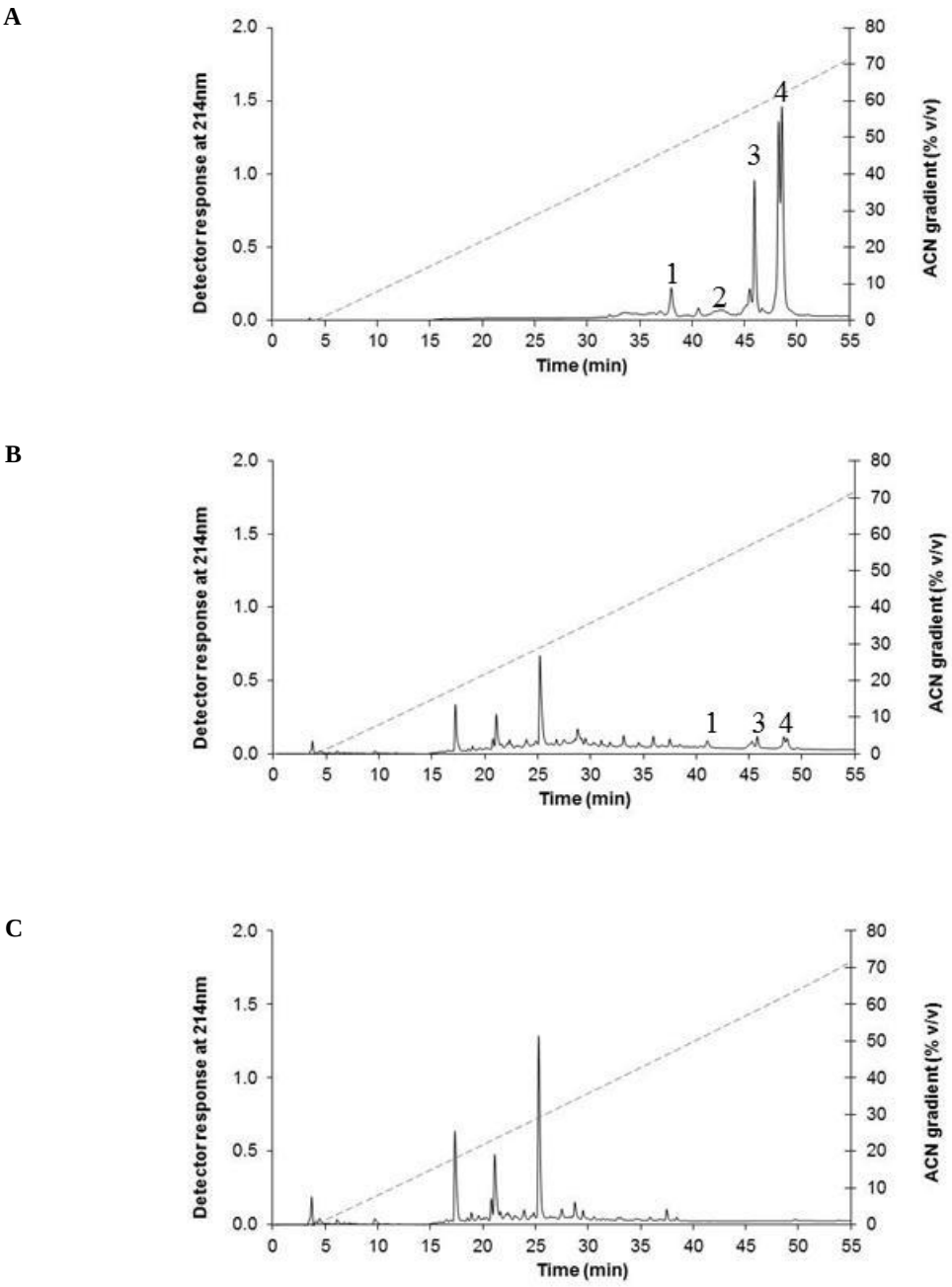
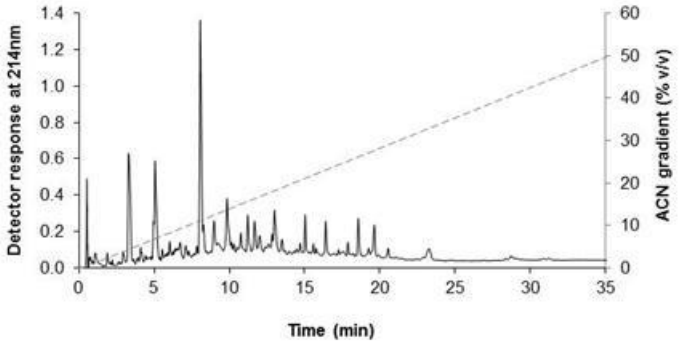
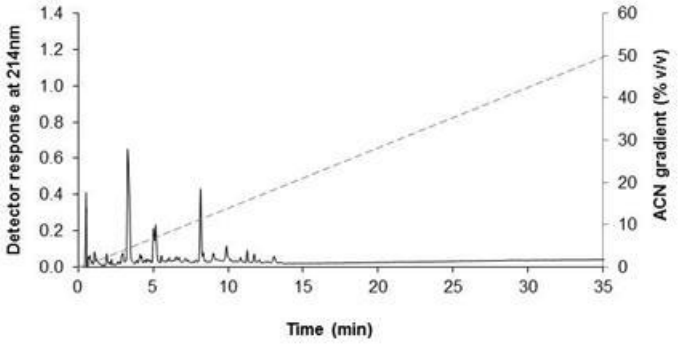


Fig. S2. Reverse-phase high pressure liquid chromatography (RP-HPLC) profiles of (a) whey protein substrate (WP1) (b) hydrolysate (WPH1) and (c) the 2 kDa permeate obtained by ultrafiltration of WPH1.

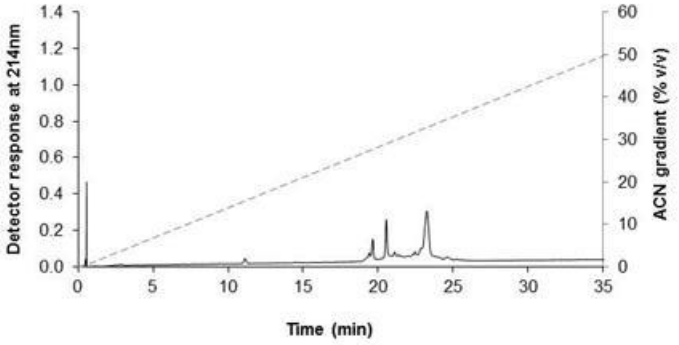
A


Detector response at 214nm
ACN gradient (% v/v)
Time (min)

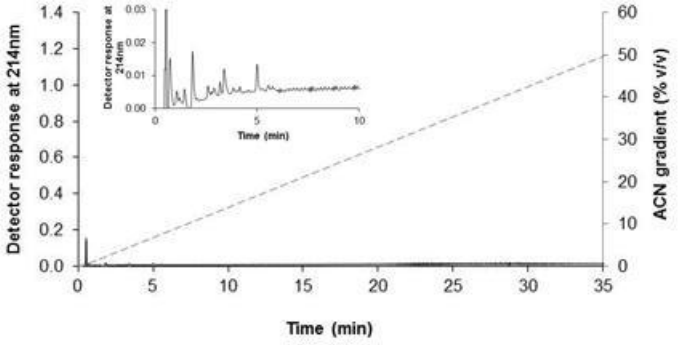
B


Detector response at 214nm
ACN gradient (% v/v)
Time (min)

C


Detector response at 214nm
ACN gradient (% v/v)
Time (min)

D


Detector response at 214nm
ACN gradient (% v/v)
Time (min)

E

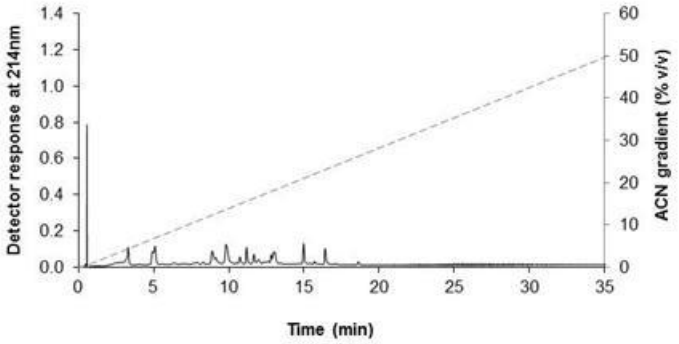

Detector response at 214nm
ACN gradient (% v/v)
Time (min)

Fig. S3. Reverse-phase ultra-performance liquid chromatography (UPLC) profiles of the whey protein hydrolysate WPH1 and insulinotropic fractions obtained by solid-phase extraction (SPE). (a) WPH1 (b) fraction unbound to SPE Strata-X cartridge (c) fraction eluted from SPE Strata-X cartridge with 40 % ACN (d) fraction eluted from SPE activated carbon (AC) cartridge with 5 % ACN and (e) fraction eluted SPE AC cartridge with 40 % ACN.