Amino Acids (2009) 37:333–339
DOI 10.1007/s00726-008-0156-0

ORIGINAL ARTICLE

Human insulinotropic response to oral ingestion of native and hydrolysed whey protein

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Received: 27 February 2008 / Accepted: 16 July 2008 / Published online: 5 August 2008

Abstract The insulinotropic response to the ingestion of whey protein and whey protein hydrolysate, independent of carbohydrate, is not known. This study examined the effect of protein hydrolysis on the insulinotropic response to the ingestion of whey protein. Sixteen healthy males ingested a 500 mL solution containing either 45 g of whey protein (WPI) or whey protein hydrolysate (WPH). The estimated rate of gastric empting was not altered by hydrolysis of the protein [18 (3) vs. 23 (3) min, $n = 16$; $P = 0.15$]. Maximum plasma insulin concentration (C_{max}) occurred later (40 vs. 60 min) and was 28% [234 (26) vs. 299 (31) mM, $P = 0.018$] greater following ingestion of the WPH compared to the WPI leading to a 43% increase [7.6 (0.9) vs. 10.8 (2.6) nM, $P = 0.21$] in the AUC of insulin for the WPH. Of the amino acids with known insulinotropic properties only Phe demonstrated a significantly greater maximal concentration [C_{max}; 65 (2) vs. 72 (3) μM, $n = 16$; $P = 0.01$] and increase (+22%) in AUC following ingestion of the WPH. In conclusion, ingestion of whey protein is an effective insulin secretagogue. Hydrolysis of whey protein prior to ingestion augments the maximal insulin concentration by a mechanism that is unrelated to gastric emptying of the peptide solution.

Keywords Amino acids · Insulin · Whey protein · Peptide hydrolysate · Gastric emptying

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Abbreviations

AA	Amino acid
EAA	Essential amino acid
Leu	Leucine
Ile	Isoleucine
Val	Valine
Phe	Phenylalanine
Lys	Lysine
Arg	Arginine
BCAA	Branched chain amino acids
GE	Gastric emptying
WPI	Whey proteins isolate
WPH	Whey protein hydrolysate
RP-HPLC	Reverse phase high performance liquid chromatography
AUC	Area under the curve
$T_{50\%}$	Half-time of gastric emptying
T_{max}	Time of maximum plasma concentration
C_{max}	Maximum plasma concentration
GIP	Glucose dependent insulinotropic polypeptide
GLP-1	Glucagon-like-peptide 1

Introduction

Dietary protein is important for health (Wolfe 2006) but the insulinotropic action of proteins and their component amino acids (AA) is somewhat underappreciated. The insulinotropic response to dietary protein may prove beneficial in the prevention and/or management of clinical conditions such as sarcopenia (Bennet et al. 1990), obesity (Halton and Hu 2004; Holt et al. 1996) and type 2 diabetes (Manders et al. 2006).

The essential amino acids (EAA) are the primary regulators of the protein-mediated insulin response (Floyd

et al. 1966) and are heterogeneous in their insulinotropic potency. Expressing the insulinotropic action of AA relative to glucose is an effective means of evaluating insulinotropic potency. Measured by the maximum plasma insulin concentration (C_{max}), a 30 min intravenous (i.v.) infusion confirmed leucine (Leu; C_{max} 193 pM), lysine (Lys; C_{max} 358 pM), phenylalanine (Phe; C_{max} 172 pM) and arginine (Arg; C_{max} 567 pM) to be the most effective insulin secretagogues. Compared to i.v. administration of glucose (100%), the insulinotropic action of Leu was ~50%, Lys 100%, Phe 48% and Arg 150%. However, a mixture of EAAs proved most effective, the insulinotropic action of the mixture was 350% of the glucose response (Floyd et al. 1966).

The milk protein, whey, is a rich source of EAAs. Addition of whey protein to a carbohydrate drink (Van Loon et al. 2000) enhances the insulin response to that attained by ingestion of carbohydrate alone. Furthermore, addition of a whey protein hydrolysate (WPH), rather than the intact protein, to a carbohydrate drink exerts an even greater insulin response (Calbet and MacLean 2002). Until recently the insulinotropic action of native and hydrolysed protein, independent of carbohydrate, was unexplored. However, emerging data suggest that the insulinotropic response is dependent on the both the source (whey vs. soy) and degree of hydrolysis of the protein used (Claessens et al. 2008). The mechanism by which ingestion of protein, protein hydrolysate or AAs, increase insulin secretion is, as yet, equivocal. Putative mechanism(s) of action include a change in gastric emptying (Calbet and MacLean 2002), the magnitude of change in circulating EAAs (Floyd et al. 1970) and the secretion of incretin hormones from the gut (Holst and Gromada 2004).

The objective of this study was to determine the insulinotropic response following ingestion of native and hydrolysed whey protein and to evaluate whether a difference exists in two of the primary regulators of this response, i.e. the rate of GE and the concentration of AAs of known insulinotropic potency.

Materials and methods

Subjects

Sixteen healthy males with no history of gastrointestinal disorders participated in the study (Table 1). All subjects were informed of the purpose and potential risks of the experimental procedures before providing written consent. All experimental protocols were approved by the University of Limerick Research Ethics Committee (ULREC 04/55).

Table 1 Subject characteristics ($n = 16$)

	Mean (SEM)	Range (min–max)
Height (m)	1.9 (0.03)	1.8–2.0
Body mass	79.7 (2.79)	68.7–92.0
Age (year)	22.4 (0.48)	21.0–25.0
BMI (kg m^{-2})	23.2 (0.6)	21.2–25.8

Experimental procedure

Subjects completed two trials in a randomised order separated by a period of 7 days. Following an overnight fast, subjects were seated in a comfortable position and a cannula inserted into a superficial arm vein that was maintained patent by saline infusion. While subjects remained seated, pre and postprandial blood samples (2.5 mL) were withdrawn as per the time series depicted in Fig. 1. All blood samples were placed in pre-chilled EDTA tubes, centrifuged (2000 *rcf*, 4°C for 10 min) and the supernatant stored at −80°C until analysed.

Test solutions

Two test solutions of 500 mL were delivered at room temperature and consumed within 5 min. Both solutions contained whey protein, either Isolac® whey protein isolate (WPI; Carbery Food Ingredients, Ireland) or Optipep 80™ whey protein hydrolysate, 30% DH (WPH; Carbery Food Ingredients, Ireland). The volume, protein content, energy density and EAA content were similar in both treatments (Table 2). Paracetamol™ was added at a fixed 20 mg kg^{-1} body mass to estimate the rate of GE (Clements et al. 1978). The validity of the Paracetamol™ technique for estimation of the rate of GE has been established by comparison with the scintigraphic technique (Naslund et al. 2000). A test re-test repeatability study ($n = 8$) of this procedure was performed and analysed by the Bland

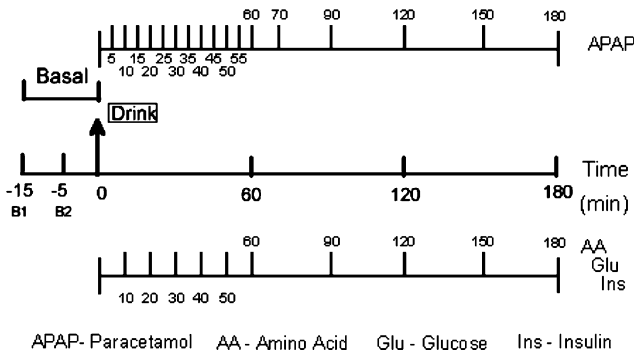

Fig. 1 Experimental protocol

Table 2 Composition of the test solutions

Composition	Whey protein isolate	Whey protein hydrolysate
Volume (mL)	500	500
Paracetamol (mg kgbm^{-1})	20	20
Protein (g L^{-1})	90	90
Molecular weight profile		
>5 kDa	83	4
>1 and <5 kDa	17	13
≤1 kDa	0	93
EAA (g L^{-1})	41	42
Energy (kJ L^{-1})	1,530	1,530

Altman technique (Bland and Altman 1986). The coefficient of repeatability (2 SD of the mean difference) of the estimated rate of GE by the Paracetamol™ technique was 7%.

Analysis

Plasma acetaminophen (Paracetamol™) concentration was measured by reversed-phase high performance liquid chromatography (RP-HPLC) using a modification of two previously published procedures (Gotelli et al. 1977, Price et al. 1983). Briefly, acetaminophen was extracted from plasma with diethyl ether, vortexed, centrifuged and the organic layer removed, dried and reconstituted in methanol. Caffeine was employed as the internal standard and separation was achieved using RP-HPLC. A temperature controlled, C_{18}-bonded silica column (Eclipse C_{18}; Agilent Technologies, CA, USA) was eluted with a mobile phase comprising ammonium acetate buffer: acetonitrile (85:15) @ 1.2 mL min^{-1}. Compounds of interest were detected by UV @ 244 nm and quantified as AUC and by reference to standards of known concentration. Analytical performance was established by repeated assay of pooled plasma samples spiked with acetaminophen and caffeine. Intra and inter assay CV was 4.4 and 9%, respectively. An estimate of GE (percentage of the meal remaining in the stomach) was computed according to the algorithm of Medhus et al. (1999) to provide the half-time of gastric emptying ($T_{50\%}$).

Perchloric acid (4:1, 0.4 M $HClO_4$:plasma) extracts of AAs were measured by pre-column derivatisation with *o*-phthalaldehyde (OPA) and quantified by RP-HPLC using a modification of the procedures described by Turnell and Cooper (1982) and Dorresteijn et al. (1996). Briefly, plasma AAs were derivatised with OPA/2-mercaptoethanol and separated by gradient elution RP-HPLC (temperature controlled). A C_{18}-bonded silica column (Gemini C_{18}, Phenomenex, CA, USA) was eluted by 2-solvent gradient comprising 20:6.5:2:71.5 propionic acid:acetonitrile:DMSO:distilled water and 40:30:7:20 acetonitrile:methanol:DMSO:distilled water. OPA derivatives were detected by fluorescence (Ex_{330}, Em_{450} nm). Norvaline was employed as the internal standard. AA derivatives were identified by retention time relative to the reference peak and quantified as AUC and by reference to standards of known concentration. Analytical performance was established by repeated assay of pooled perchloric extracts spiked with AAs of known concentration. The intra-assay precision was estimated by assaying 15 pooled plasma extracts supplemented with AAs. Mean (SD) analytical recovery was 110 (6), 99 (0.7) and 104 (2.5)% for low, medium and high concentration and CV for recovery of all AAs was <6%. Inter-assay precision was established from 15 replicate analysis of a pooled plasma extract supplemented with AA. Three aliquots were analysed per day over 5 days. Mean (SD) analytical recovery was 106 (7), 100 (0.9) and 104 (3.4) % for low, medium and high concentration and CV for recovery of all AAs was ≤10%.

Plasma insulin was measured by radioimmunoassay (RIA; DSL 1600, Texas, USA). Intra- and inter-assay CV, determined from repeated assay of a pooled plasma sample, was 6.6 and 7%, respectively. Plasma glucose concentration was measured by automated glucose analyser (Analox GM7, London, UK). Intra-assay CV of a pooled plasma sample was 2.1% and inter-assay CV was 7%.

Statistical analysis

All data are presented as the mean (SEM, $n = 16$). Data were tested for normality (Shapiro-Wilk W). A two-factor (Treatment × Time) analysis of variance (ANOVA) was used with repeated measures on the factor Time. Where significant interaction was evident a post hoc paired sample *t* test with Bonferroni correction was employed to examine the differences at key time points. Area under the curve (AUC) above baseline for each analyte was calculated by trapezoidal integration and difference in AUC between treatments was analysed by paired-sample *t* test. The effect of hydrolysis on the peak concentration (identified from the mean profiles) and on mean maximal plasma concentration (C_{max}) of each analyte was examined. The difference in peak concentration and C_{max} between treatments was analysed by paired-sample *t* test. WPI data were used as the control against which differences due to protein hydrolysis (WPH) was compared. Stepwise multiple linear regression was applied to assess which AA, alone or in combination, was the best predictor of the plasma insulin response. Statistical significance was set at $P < 0.05$. All analyses were performed using SPSS (v.15, SPSS Inc, Chicago, IL).

Results

Insulinotropic response

Resting plasma insulin values were similar for the WPI and WPH [95 (4) vs. 100 (5) pM, $n = 16$; $P = 0.61$). Following protein ingestion, a large range in both the magnitude (C_{max}; range 135–565 pM) and timing (T_{max}; range 20–60 min) of the subjects' maximum plasma insulin response, C_{max}, was observed indicating a highly variable within-subject response. $C_{max,}$ was significantly greater (+28%) following ingestion of the hydrolysed whey protein [234 (26) vs. 299 (31) pM, $n = 16$, $P = 0.018$: Fig. 2a inset). Plasma insulin increased rapidly in the initial 30 min of the postprandial period for both trials. However, mean plasma insulin peaked later and at a higher concentration, in the WPH trial (40 vs. 60 min and 194 (12) vs. 234 (35) pM, $n = 16$; $P = 0.2$: Fig. 2). Though not statistically significant, when analysed over the 3 h period of observation, the AUC for insulin was 43% greater following ingestion of the hydrolysed compared to the intact whey protein [7.6 (0.9) vs. 10.8 (2.6) nM 3 h^{-1}, $n = 16$; $P = 0.21$].

Plasma glucose

Fasting blood glucose concentration did not differ between the WPI and WPH trials. Analysed over the 40–90 min postprandial period, mean blood glucose decreased by a maximum of 0.7 mM in both the WPI [4.0 (0.1) to 3.3 (0.2) mM, $n = 16$; $P < 0.001$] and WPH [3.8 (0.1) to 3.1 (0.1) mM, $n = 16$; $P < 0.001$) trials (Fig. 2b). Plasma glucose concentration returned to near basal levels in the final hour of the postprandial period.

Gastric emptying

The estimated time-dependent rate of GE for the whey protein solutions is depicted in Fig. 3. Mean estimated half-time of gastric emptying, $T_{50\%}$, of the hydrolysed (WPH) protein was found to be not significantly different [18 (3) vs. 23 (3) min, $n = 16$; $P = 0.15$] from the native protein (WPI). At 120 min the WPI and WPH had completely emptied from the stomach at an average rate of emptying of ~6.4 kJ min^{-1}.

Branched chain amino acids

Plasma branched chain amino acids (BCAA) is a summation of leucine (Leu) isoleucine (Ile) and valine (Val).

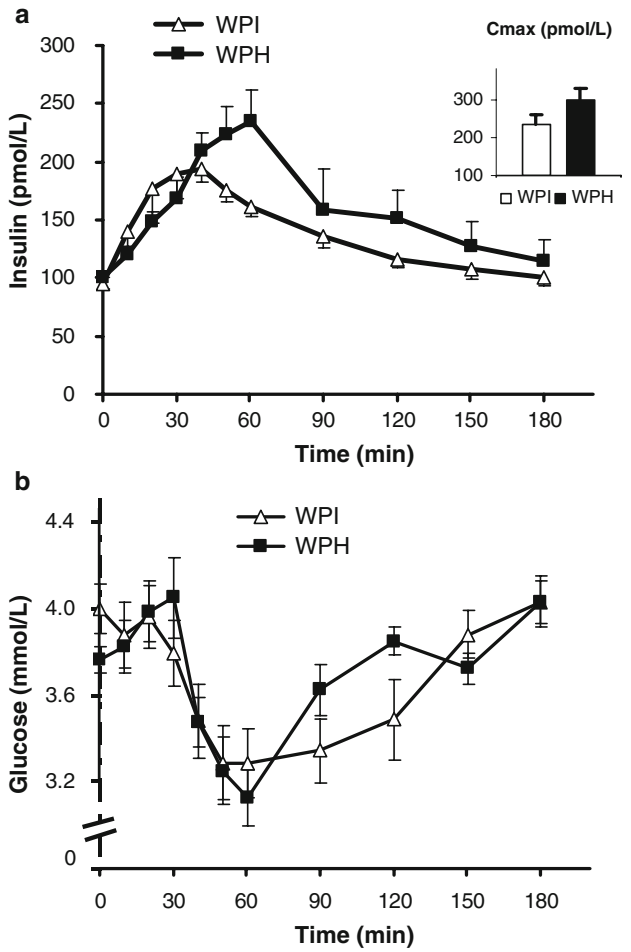

Fig. 2 Mean plasma insulin and glucose concentration for the whey protein isolate (*WPI*) and whey protein hydrolysate (*WPH*) during the postprandial period. *Inset* denotes mean maximum plasma insulin concentration (C_{max}). Values are mean (SEM) $n = 16$

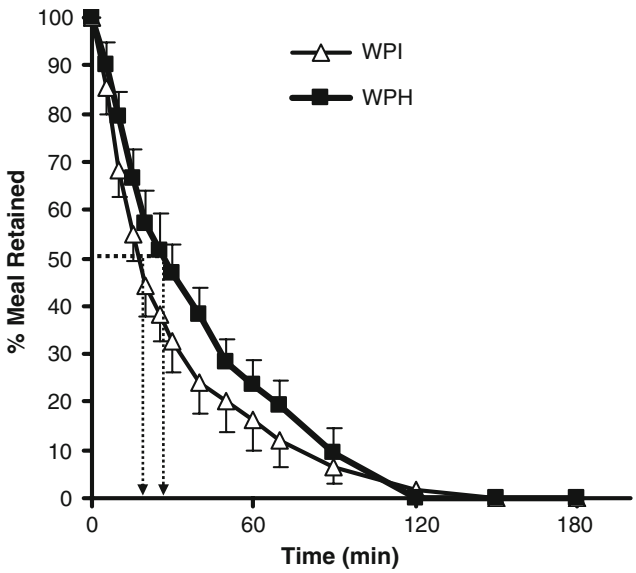

Fig. 3 Gastric emptying for the whey protein isolate (*WPI*) and the whey protein hydrolysate (*WPH*) expressed as the percentage of the meal retained in the stomach with respect to time. Values are mean (SEM) $n = 16$

Mean basal plasma BCAA concentration was identical in the WPI and WPH trials [294 (16) vs. 295 (13) μM, $n = 16$; $P = 0.93$]. There was no difference in the rate of appearance of plasma BCAAs in the initial 40 min of the postprandial period and the T_{max} for the WPI and WPH was 60 min (Fig. 4). However, mean peak concentration [−8%; 843 (37) vs. 790 (40) μM, $n = 16$; $P = 0.176$] and mean AUC [−15%; 69 (4) vs. 59 (5) mM 3 h^{-1}, $n = 16$; $P = 0.07$] were lower in the WPH compared to the WPI trial. The mean BCAA C_{max} was also lower in the WPH trial [−8%; 884 (40) vs. 811 (39) μM, $n = 16$ $P = 0.09$: Fig. 4 inset]. No relationship was found between the change in plasma BCAAs and plasma insulin after ingestion of the native whey protein (WPI $r = 0.56$, $P = 0.013$) and hydrolysis did not strengthen this relationship (WPH $r = -0.04$, $P = 0.5$).

Phenylalanine

At rest, mean plasma Phe concentration was similar in both WPI and WPH trials [39 (1) vs. 40 (2) μM, $n = 16$; $P = 0.86$]. Significant difference in Phe concentration was observed during the early postprandial period (30–60 min; Fig. 5), reaching peak Phe values that were 10% higher in the WPH relative to the WPI trial [62 (2) vs. 70 (3) μM, $n = 16$; $P = 0.003$]. This Phe response was very much limited to the early postprandial period as the overall 3 h AUC, though 22% higher for the WPH trial, did not reach statistical significance [2.9 (0.3) vs. 2.4 (0.4) mM 3 h^{-1}, $n = 16$; $P = 0.23$]. The mean Phe C_{max} was significantly higher after ingestion of the WPH [+10%; 65 (2) vs. 72 (3) μM, $n = 16$; $P = 0.01$: Fig. 5 inset]. Regression analysis revealed a poor correlation between the magnitude of the plasma insulin and Phe response following ingestion of native (WPI; $r = 0.12$, $P = 0.3$) and hydrolysed (WPH; $r = 0.19$, $P = 0.3$) whey protein.

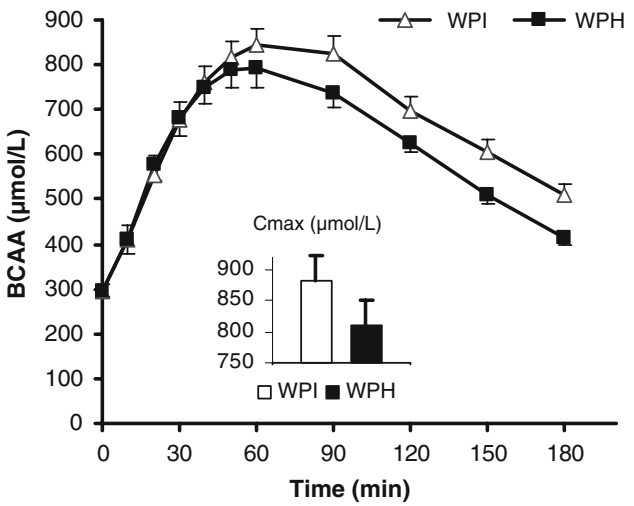

Fig. 4 Mean plasma branched chain amino acids (*BCAA*) concentration for the whey protein isolate (*WPI*) and whey protein hydrolysate (*WPH*) during the postprandial period. *Inset* denotes mean maximum plasma insulin concentration (C_{max}). Values are mean (SEM) $n = 16$

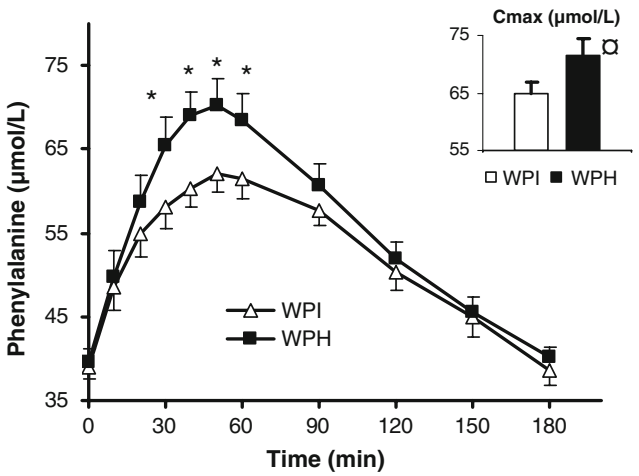

Fig. 5 Mean plasma phenylalanine concentration for the whey protein isolate (*WPI*) and whey protein hydrolysate (*WPH*) during the postprandial period. *Inset* denotes mean maximum plasma insulin concentration (C_{max}). Values are mean (SEM) $n = 16$. * $P < 0.05$ versus WPI and ¤ $P < 0.05$ versus WPI

Discussion

The primary objective of this study was to assess the insulinotropic response to ingestion of native and hydrolysed whey protein in healthy, adult males. The data presented in this paper show that ingestion of 45 g of whey protein induced a significant insulin response, the post-prandial 3 h AUC for insulin being 7.6 (0.9) and 10.8 (2.6) nM 3 h^{-1} for the WPI and WPH, respectively. Confirmation that the insulinotropic effect was independent of glucose is provided by the observation that the mean plasma glucose concentration actually decreased (by a maximum of 0.7 mM) at the time corresponding to the peak in plasma insulin (Fig. 2a, b).

The exact mechanism of insulin secretion following ingestion of whey protein is, as yet, equivocal but the rate of gastric emptying, (Calbet and MacLean 2002), change in circulating EAAs (Floyd et al. 1970) and the release of incretin hormones (Holst and Gromada 2004) are proposed as the primary regulators. In the present study, similar half-time rates of gastric emptying ($T_{50\%}$), as assessed by the paracetamol technique, were observed for WPI and WPH [18 (3) vs. 23 (3) min; $P = 0.15$: Fig. 3]. The non-significantly different, rates of GE compare favourably to the $T_{50\%}$ reported earlier by Calbet and Holst (2004), using a double sampling aspiration technique, for a native and hydrolysed whey protein [19 (3) vs. 21 (1) min; $P > 0.05$].

The rate of appearance of the BCAAs in the circulation can also act as an indirect index of GE and intestinal absorption (Calbet and MacLean 2002) as BCAAs are relatively unaffected by gut metabolism and hepatic uptake (Wahren et al. 1976). As shown in Fig. 4 the rate of appearance of BCAAs was not significantly altered by hydrolysis of the native whey protein. In combination, these data confirm that, when matched for volume and total protein, both the native and hydrolysed form of the soluble whey protein used in this study emptied from the stomach at the same rate.

Amino acid-induced insulin secretion is principally confined to the essential amino acids (EAAs) and to the change in their circulating concentration. In this respect, secretion of insulin from pancreatic β-cells can be increased by infusion (Floyd et al. 1966) or ingestion (Van Loon et al. 2000) of EAAs. Whey protein is composed of between 40 and 50% EAAs and is considered a rich source of these insulin regulatory AAs. In the present study, stepwise linear regression was used to evaluate the relationship between the change in AAs of known insulinotropic potency and the resultant insulin response. Of the insulin-stimulating AAs only Phe showed a significantly greater maximal concentration (C_{max}; Fig. 5a inset) and a 22% increase in AUC following ingestion of the WPH compared to WPI (Fig. 5). However, the weak and statistically insignificant, relationship between plasma Phe and insulin for the native (WPI; $r = 0.12$, $P = 0.3$) or hydrolysed (WPH; $r = 0.19$, $P = 0.3$) whey protein would not support the change in the circulating concentration of Phe as the primary regulator of the increase in insulinotropic response. Stepwise addition of other insulinotropic AAs to the analysis did not improve the power of the prediction. In contrast to these data, previous studies have shown a highly significant relationship between Phe and the insulin response (Calbet and MacLean 2002; Van Loon et al. 2000) or have attributed the whey-induced hyperinsulinemia to the insulin stimulating effects of the BCAAs, lysine and threonine (Nilsson et al. 2007), but all these studies were undertaken with carbohydrate (glucose) added to a whey protein or protein hydrolysate. In the absence of carbohydrate, the present study found no relationship between the change in plasma BCAA and insulin concentration for either the native (WPI; $r = 0.56$, $P = 0.013$) or hydrolysed whey protein (WPH; $r = -0.04$, $P = 0.5$). Given the multiple pathways of activation of insulin secretion from the pancreatic β-cell (Newsholme et al. 2007) it is probable that Phe and BCAAs act synergistically with glucose to augment pancreatic insulin secretion following ingestion of protein and carbohydrate.

Though it is widely accepted that a change in the circulating EAAs is the primary regulator of the postprandial insulin response, accounting for at least 60% of the change in plasma insulin (Schmid et al. 1992), it is important to recognise that hydrolysis of whey protein can also influence the secretion of incretin hormones, such as glucose dependent insulinotropic polypeptide (GIP) and glucagon-like-peptide 1 (GLP-1) (Frid et al. 2005). To this effect, Nilsson et al. (2007) recently reported a significantly greater (+50%) GIP response to ingestion of whey protein than to a comparable mixture of AAs. Furthermore, hydrolysis of whey protein is reported to induce a greater GIP response than the intact protein (Calbet and Holst 2004). The incretin hormone response may therefore be significant, but in neither study was it possible to apportion the insulin-stimulatory effect of the increased GIP response.

In conclusion, the feeding of 45 g of whey protein was found to be an insulin secretagogue. The resultant hyperinsulinemia was found to be glucose independent and, on the basis of a 28% increase in maximal plasma insulin concentration (C_{max}), was augmented by hydrolysis of the protein. The insulinotropic potency of hydrolysed whey protein could provide nutraceutical benefit in clinical settings where the glucose sensing capacity of the pancreatic β-cell is reduced i.e. type 2 diabetes (Manders et al. 2006).

Acknowledgments The authors acknowledge the financial support of Enterprise Ireland (Grant IP 2004-237) and Carbery Food Ingredients.

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