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ARTICLE

Comparison of Different Sources and Degrees of Hydrolysis of Dietary Protein: Effect on Plasma Amino Acids, Dipeptides, and Insulin Responses in Human Subjects

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The effect of protein fractionation on the bioavailability of amino acids and peptides and insulin response and whether the protein source influences these effects in humans are poorly understood. This study compared the effects of different sources and degrees of hydrolysis of dietary protein, independent of carbohydrate, on plasma amino acid and dipeptide levels and insulin responses in humans. Ten subjects were enrolled in the study, with five subjects participating in trials on either soy or whey protein and their hydrolysates. Protein hydrolysates were absorbed more rapidly as plasma amino acids compared to nonhydrolyzed protein. Whey protein also caused more rapid increases in indispensable amino acid and branched-chain amino acid concentrations than soy protein. In addition, protein hydrolysates caused significant increases in Val-Leu and Ile-Leu concentrations compared to nonhydrolyzed protein. Whey protein hydrolysates also induced significantly greater stimulation of insulin release than the other proteins. Taken together, these results demonstrate whey protein hydrolysates cause significantly greater increases in the plasma concentrations of amino acids, dipeptides, and insulin.

KEYWORDS: Whey protein hydrolysates; plasma amino acids; plasma peptides; insulin response

INTRODUCTION

Both the source and degree of hydrolysis of dietary proteins are known to influence their absorption rate. Proteins are grouped according to source as either quickly or slowly digestible proteins (*1*−*3*). Quickly digestible proteins, such as whey protein, are soluble and rapidly digested and absorbed, whereas slowly digestible proteins, such as casein, form clots in the stomach, resulting in slower gastric emptying and intestinal absorption (*1*, *4*, *5*). Soy protein is digested in a manner more similar to whey protein than to casein (*6*). The chain length of the peptides also has an important influence on the absorption rate of partially hydrolyzed proteins. Several in vitro studies in humans have shown that a nitrogen load is absorbed more rapidly when delivered as di- or tripeptides rather than as whole protein or free amino acids (*7*−*12*). However, a more rapid increase in plasma amino acids after oral feeding of hydrolysates compared to whole protein has been described for only casein in human subjects (*5*, *13*).

Studies on protein digestion in the human small intestine have revealed the main products of protein digestion in the gut lumen are not single amino acids, but rather di- and tripeptides (*7*, *8*). The intestinal oligopeptide transporter Pept-1 was cloned in the intestines of humans and experimental animals (*14*, *15*). This led ultimately to the demonstration that di- and tripeptides are actively and rapidly taken up by enterocytes via Pept-1. Bioactive peptides are therefore attracting increasing interest in the development of functional food products. The most studied class of bioactive peptides are those with blood pressure-lowering activity (*16*). Foltz et al. (*17*) demonstrated that angiotensin converting enzyme inhibitory peptides are absorbed into the circulation as intact forms. A small number of nutritional studies showed dietary peptides are absorbed as intact forms by quantifying their levels in the circulation. Recently, we identified branched-chain amino acid (BCAA)-containing dipeptides in whey protein hydrolysates that included Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu, and Leu-Leu. Using L6 myotubes, these peptides were shown to stimulate glucose uptake in skeletal muscle (*18*). We therefore speculated that the plasma levels of these peptides may be elevated markedly after ingestion of protein hydrolysates compared to nonhydrolyzed protein.

It is well-known that dietary proteins co-ingested with carbohydrate induce insulin secretion in healthy and diabetic subjects (*19*−*24*). Nilsson et al. (*25*) demonstrated that milk proteins have insulinotropic properties, with the whey fraction being a more efficient insulin secretagogue than casein when ingested with carbohydrate. Van Loon et al. (*24*) demonstrated that co-ingestion of carbohydrate and protein hydrolysates, rather than

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intact protein, was the preferred option for stimulating insulin secretion. Almost all research in this area has compared the results with the insulinotropic effect of dietary protein and protein hydrolysates from different sources co-ingested with carbohydrate. Until recently, the insulinotropic action of intact protein and hydrolyzed protein, independent of carbohydrate, remained unexplored. Claessens et al. (*26*) then demonstrated that the insulinotropic response was dependent on both the source (whey or soy) and degree of hydrolysis of the protein used. However, it remains equivocal whether ingestion of protein and protein hydrolysates without carbohydrate increases insulin levels to the same extent as when ingested with carbohydrate.

The amino acid profile of the ingested protein also plays a role in the hormonal response to feeding. For example, some amino acids such as arginine, lysine, phenylalanine, alanine, leucine, and isoleucine stimulate insulin release (*27*−*31*). We hypothesized that the postprandial levels of amino acids and dipeptides and composition of plasma amino acids and dipeptides may have a beneficial effect by enhancing insulin secretion.

Several studies have demonstrated that skeletal muscle protein synthesis (*32*) and glycogen accumulation (*33*,*34*) are induced not only by insulin but also by certain amino acids (*35*). The ability of insulin and amino acids to promote muscle protein anabolism and/or muscle glycogen synthesis in sport settings, and also to improve hyperglycemia levels in the clinical setting, has led to increasing interest in nutritional strategies that maximize insulin levels and increase the availability of plasma amino acids and dipeptides. The aim of the current study was therefore to compare the effects of different sources and hydrolysis of dietary proteins on plasma concentrations of amino acids and BCAA-containing peptides in human subjects. A further objective was to determine the effect of these proteins on insulin levels and which amino acids specifically influenced production of the hormone.

MATERIALS AND METHODS

Subjects. Ten subjects participated in the study. The 10 subjects (4 females and 6 males) were assigned randomly into two groups according to body weight. Five subjects (2 females and 3 males) participated in trials on soy protein and its hydrolysates and the other five subjects participated in trials on whey protein and its hydrolysates. The subjects in the soy group had a mean (±SEM) age of 37 ± 3 years, mean weight of 59.5 ± 4.1 kg, and mean height of 166 ± 2 cm, whereas the whey group had a mean age of 33 ± 4 years, mean weight of 63.5 ± 4.0 kg, and mean height of 166 ± 2 cm. There was no difference in these parameters between the two groups. This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the ethics committee of Meiji Seika Kaisha Ltd., Food and Health R&D Laboratories. Written informed consent was obtained from all subjects prior to enrollment in the study.

Experimental Design. *Sample Preparation.* Soy protein, soy protein hydrolysates (Fuji Oil Co., Ltd., Japan), whey protein (Fonterra Co-operative Group Ltd., New Zealand), and whey protein hydrolysates (Tatua Co-Operative Dairy Co. Ltd., New Zealand) were used in this study. Protein hydrolysates were obtained by enzymatic hydrolysis.

The amino acid content of the hydrolyzed proteins was analyzed by an amino acid analyzer using ion-exchange chromatography (*36*). The protein content of the samples was calculated from the sum of amino acid contents and was 85.5% for soy protein, 86.1% for soy protein hydrolysates, 83.8% for whey protein, and 86.9% for whey protein hydrolysates. The amino acid composition of the experimental diets is shown in **Table 1**.

BCAA-containing dipeptides in the protein samples were assayed by LC-MS/MS (*18*). This demonstrated that these peptides were not detected in either soy or whey nonhydrolyzed protein. Val-Leu, Ile-Leu, and Leu-Leu were the main components in both soy and whey protein hydrolysates, whereas Val-Val and Val-Ile were not detected (**Table 1**). The amino acid sequences of the BCAA-containing dipeptides from whey protein [β-lactoglobulin (GI:7766897), α-lactalbumin (GI:12084472)], and soy protein [glycinin (GI:33357661)] are shown in **Table 2**.

The average chain length of the peptides was calculated as the ratio of total nitrogen content (TN) minus the nitrogen content of amino acid side chains to the amino nitrogen content (AN) in the protein samples. TN and AN were assayed using the methods of Kjeldahl and van Slyke, respectively. Average peptide length was similar in the soy (TN/AN = 3.45) and whey protein hydrolysates (TN/AN = 3.01). The molecular weight distribution was analyzed using gel filtration HPLC on a TSKgel G2500PW column (7.5 × 300 mm, Tosoh, Inc., Japan). A mobile phase of 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) was utilized at a flow rate of 0.4 mL/min. Molecular mass standards aprotinin

Table 1. Amino Acid Composition (g/100 g of Protein) and Free Dipeptide Content (g/100 g of Protein) in the Protein Samples[a]

	soy		whey	
	protein	hydrolysates	protein	hydrolysates
Amino Acid Composition				
Ala	4.13	4.10	5.08	5.09
Arg	7.60	7.99	2.33	2.73
Asx	11.7	12.3	10.6	10.4
Cys	1.30	1.16	2.47	2.27
Glu	19.5	21.4	16.8	17.1
Gly	4.13	4.24	1.85	1.77
His	2.63	2.72	1.90	2.11
Ile	4.48	3.73	6.03	5.64
Leu	7.81	6.62	10.2	12.3
Lys	6.16	6.54	9.00	9.63
Met	1.23	1.09	2.08	2.09
Phe	5.25	4.52	3.06	3.72
Pro	5.37	5.54	5.96	5.04
Ser	5.24	5.47	5.06	4.39
Thr	3.83	3.90	7.08	4.91
Trp	1.29	1.06	1.83	1.84
Tyr	3.74	3.54	2.98	3.53
Val	4.56	4.12	5.60	5.40
IAA	34.6	31.6	44.9	45.4
BCAA	16.9	14.5	21.9	23.3
Free Dipeptide Content				
Ile-Val	nd	0.95	nd	0.24
Leu-Val	nd	0.29	nd	0.28
Val-Leu	nd	2.38	nd	6.58
Ile-Ile	nd	0.13	nd	0.08
Leu-Ile	nd	0.03	nd	0.02
Ile-Leu	nd	1.70	nd	3.29
Leu-Leu	nd	3.26	nd	1.48

[a] Asx, Asp + Asn; Glx, Glu + Gln; IAA, indispensable amino acids; BCAA, branched-chain amino acid. nd, not detected.

Table 2. Amino Acid Sequence of BCAA-Containing Dipeptides from Whey Protein (β-Lactoglobulin, α-Lactalbumin) and Soy Protein (Glycinin)

$[M + H]^+$	sequence	origin	fragment
231.16	Val-Leu	β-lactoglobulin	92−93
			94−95
		glycinin	121−122
			465−466
245.18	Ile-Leu	β-lactoglobulin	56−57
		α-lactalbumin	95−96
	Leu-Leu	β-lactoglobulin	31−32
			57−58
			103−104
		glycinin	145−146
			420−421

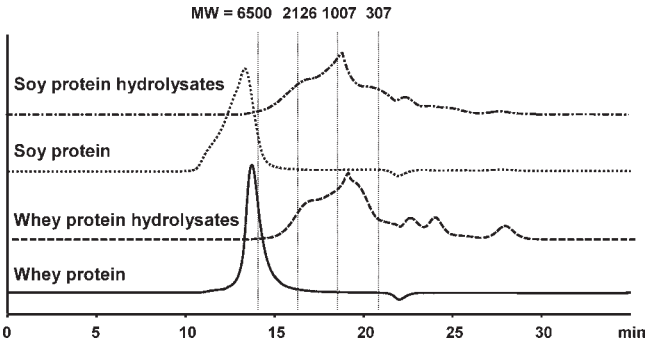

Figure 1. Molecular weight distribution in the protein samples.

(MW = 6500, Sigma-Aldrich, St. Louis, MO), gastin (MW = 2126, Sigma-Aldrich), oxytocin (MW = 1007, Sigma-Aldrich), and glutathione (MW = 307, Wako Pure Chemical Industries, Osaka, Japan) were separated on the same column under similar HPLC conditions. A chromatogram of the molecular weight distribution is shown in **Figure 1**.

Protocol. The subjects were requested to fast for at least 12 h and then report to the laboratory. The subjects then ingested a test drink containing 12.5 g of protein [7% solution (w/v)] as quickly as possible within 3 min. Blood samples were drawn from the antecubital vein before ($t = 0$) and 10, 20, 30, 45, 60, 90, and 120 min after consumption of the test solution.

Blood Analysis. Blood was collected into EDTA-containing tubes for plasma amino acid and peptide analysis. After collection, the blood samples were centrifuged at 1000*g* at 4 °C for 15 min and the plasma samples stored at −80 °C until assayed. Plasma free amino acids were determined by HPLC, with precolumn 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate derivatization (*37*). Plasma proteins were removed with 5% trichloroacetate, and after neutralization, the samples of plasma free amino acids were subjected to HPLC.

Plasma glucose concentrations were measured using a glucose oxidase assay (*38*), insulin concentration was determined by an ELISA kit obtained from Mercodia, Uppsala, Sweden, and glucagon level was established by a RIA kit (Daiichi Radioisotope, Tokyo, Japan).

Identification of BCAA-Containing Dipeptides in Human Plasma. Plasma proteins were removed with 5% trichloroacetate and the samples subjected to HPLC. BCAA-containing dipeptides were identified using a LC-MS/MS system (Quatro premier XE, Waters Corp., Milford, MA). All of the analyses were performed on a 2.1 × 50 mm column with a particle size of 1.7 μm (Acquity UPLC BEH C18, Waters Corp.). Mobile phase A consisted of 0.05% TFA in Milli-Q water, whereas mobile phase B consisted of 0.05% TFA in acetonitrile. The initial eluent composition was 100% A, followed by an increase to 40% B for 9 min and to 80% for 1 min and then a reduction to 100% A for 3 min. Total running time was therefore 13 min. The eluent flow was 0.3 mL/min, and the column temperature was set at 40 °C. The UV trace was recorded at 215 nm, and the analytes were detected using electrospray ionization in the positive mode. Multiple reaction monitoring (MRM) was performed using characteristic fragmentation ions (m/z 245.1 > 86.1 for Ile-Ile, Leu-Ile, Ile-Leu, Leu-Leu; m/z 231.1 > 86.1 for Ile-Val, Leu-Val; m/z 231.1 > 72.1 for Val-Leu). The parameters for the LC-MS/MS analysis of BCAA-containing dipeptides were as follows: capillary voltage, 3000 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas flow, 849 L/h; cone gas flow, 48 L/h; cone voltage, 25 V; and collision energy, 15 eV.

Statistics. Data were expressed as means ± SEM. The area under the curve (AUC) was calculated for plasma concentrations minus the baseline value ($t = 0$). Data were analyzed using two-way ANOVA that included between-subject (difference of dietary protein source) and within-subject (difference of protein hydrolysis) variation. The associations between variables were examined using Pearson's correlation coefficient. Differences between the groups were considered to be significant at $P < 0.05$. All analyses were performed using SPSS ver. 12.0 (SPSS Inc., Chicago, IL).

RESULTS

Changes in Amino Acid Concentrations. The plasma concentrations of total amino acids (TAA), indispensable amino acids (IAA), and BCAA are shown in **Figure 2**. Plasma levels of TAA increased significantly 10, 20, 30, and 45 min after ingestion of the protein hydrolysates compared to levels attained following ingestion of whole protein. However, TAA levels were significantly lower at 90 min. There was also a significant increase in plasma IAA at 10, 20, and 30 min after ingestion of protein hydrolysates compared with whole protein. In addition, the protein hydrolysate beverages caused significant increases in plasma BCAA levels at 10, 20, 30, and 40 min compared to the whole protein beverages, followed by a decrease at 90 min.

The concentration of TAA at 60 min was significantly higher in the whey protein group compared to the soy protein group. At any time point, plasma IAA concentrations were significantly lower in the soy protein group than in the whey protein group. We also observed a significant increase in plasma BCAA concentration at 20, 30, 45, 60, and 90 min in the whey protein group compared to the soy group.

When the data were expressed as AUCs for the 0−60 min period, the plasma levels of TAA ($P = 0.001$), IAA ($P < 0.001$), and BCAA ($P < 0.001$) were significantly higher in subjects given the hydrolyzed protein than in subjects given the nonhydrolyzed protein. Plasma alanine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, and valine were significantly higher in the hydrolyzed protein group than in the nonhydrolyzed protein group, whereas plasma levels of arginine and asparagine were significantly lower (**Table 3**).

The plasma levels of TAA ($P = 0.027$), IAA ($P < 0.001$), and BCAA ($P < 0.001$), calculated as AUC (0−60 min), were also significantly higher after whey protein ingestion compared to soy protein. Plasma levels of alanine, arginine, cystine, glutamic acid, isoleucine, leucine, lysine, methionine, proline, threonine, tryptophan, and valine, calculated as AUC (0−60 min), also increased significantly after whey protein ingestion compared to soy protein, whereas plasma levels of glycine were significantly decreased.

When the data were expressed as AUC for the 0−120 min period, there was no difference in plasma TAA, IAA, and BCAA between hydrolyzed protein and whole protein. Plasma levels of IAA ($P = 0.001$) and BCAA ($P = 0.001$) were significantly higher in the whey protein group than in the soy protein group. Plasma levels of isoleucine, leucine, lysine, methionine, threonine, tryptophan, and valine were significantly higher in the whey protein group than in the soy protein group (**Table 4**).

Concentration of BCAA-Containing Dipeptides. The plasma concentrations of Val-Leu, Ile-Leu, and Leu-Leu are shown in **Figure 3**. Data on the plasma levels of Ile-Val, Leu-Val, Ile-Ile, and Leu-Ile are not shown as the concentrations of these dipeptides were detected at only trace levels.

Plasma levels of Val-Leu increased significantly 10, 20, 30, and 45 min after ingestion of the protein hydrolysates compared to levels following ingestion of whole protein. There was also a significant increase in plasma Ile-Leu at 10, 20, and 30 min after ingestion of protein hydrolysates compared with ingestion of whole protein. However, levels of Leu-Leu were similar in the two groups. At 45, 60, and 90 min, there was a significant increase in plasma concentration of Val-Leu and Ile-Leu after ingestion of whey protein compared to soy protein. The Leu-Leu concentrations at 30, 45, and 60 min were also significantly higher in the whey protein group compared to the soy protein group.

Analysis of AUC for the 0−60 min period showed protein hydrolysates resulted in significantly higher levels of Val-Leu ($P = 0.031$) and Ile-Leu ($P = 0.035$) compared to nonhydrolyzed protein. The AUC for the 0−120 min period showed Val-Leu ($P = 0.048$) and Ile-Leu ($P = 0.042$) levels were significantly greater in the protein hydrolysate groups than in the whole

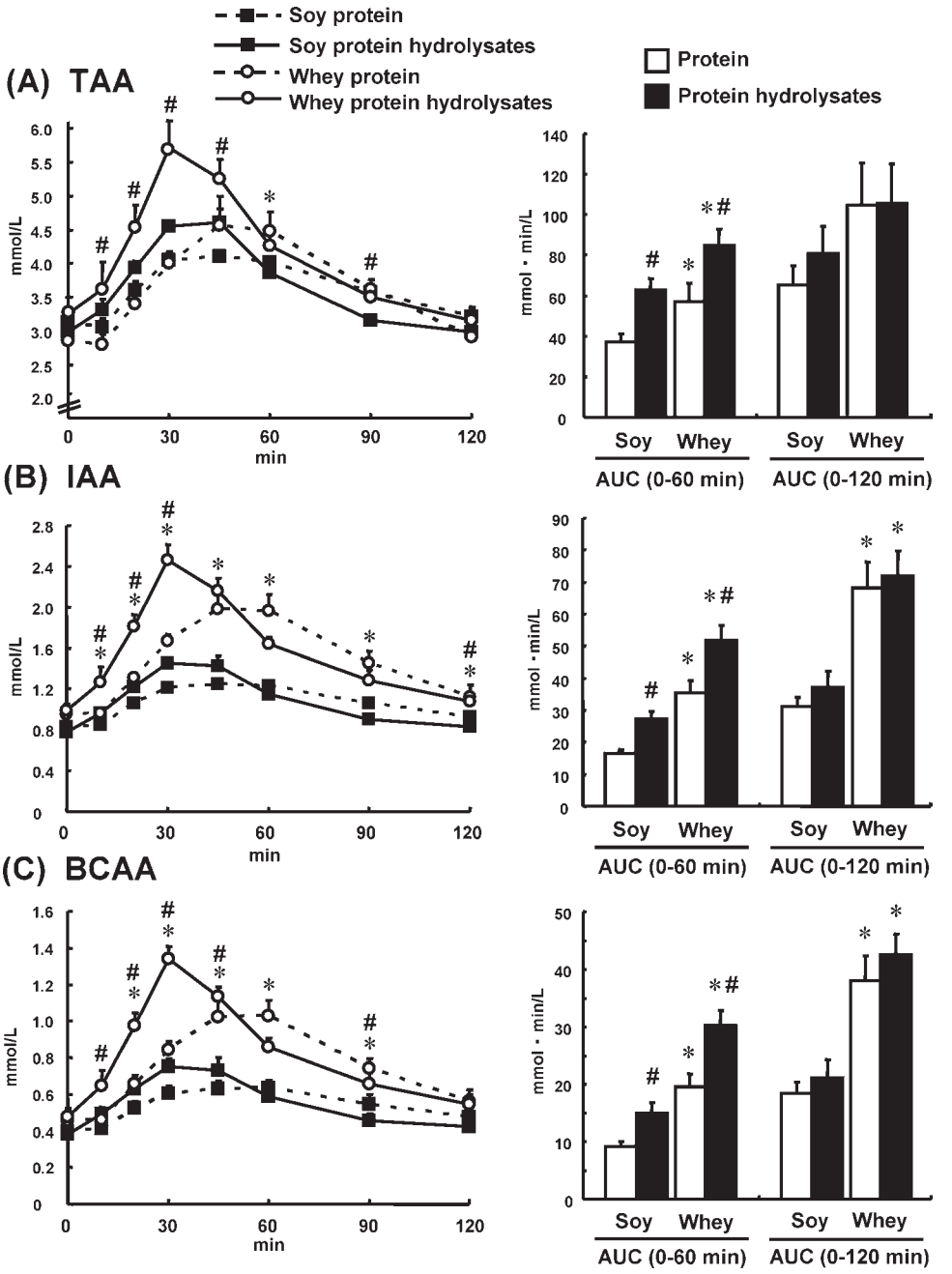

Figure 2. Plasma concentrations of (**A**) total amino acids, (**B**) indispensable amino acids, and (**C**) branched-chain amino acids. The left-hand graphs show the plasma concentrations of these compounds over the 120 min study period. The right-hand graphs show the area under the curve (AUC) for the 0−60 and 0−120 min periods. Values are expressed as means ± SEM, n = 5/group. #, $P < 0.05$ significant difference between nonhydrolyzed protein and protein hydrolysates; ∗, $P < 0.05$ significant difference between dietary protein source.

protein group. Dietary whey protein also caused significant increases in plasma levels of Ile-Leu ($P = 0.048$) compared to soy protein (**Figure 3**).

Plasma Glucose, Insulin, and Glucagon Levels. The plasma glucose concentrations were not different between the groups (**Figure 4**). The changes in plasma levels of insulin and glucagon are shown in **Figure 5**, with plasma insulin levels increasing significantly 10, 20, 30, 60, and 90 min after ingestion of the protein hydrolysates compared to whole protein. The concentration of insulin at 45 min was significantly higher in the whey protein group compared to the soy protein group. There was also a significant increase in plasma glucagon 20, 30, and 90 min after ingestion of protein hydrolysates compared with ingestion of whole protein.

When expressed as AUC (0−60 min), insulin levels were significantly higher with protein hydrolysates than with nonhydrolyzed protein ($P = 0.005$). In addition, whey protein significantly stimulated the insulin response, expressed as AUC for both the 0−60 min ($P = 0.035$) and 0−120 min ($P = 0.041$) periods, compared to soy protein. Analysis of AUC for the 0−60 min period showed whey protein resulted in significantly higher glucagon levels ($P = 0.042$) compared to soy protein. The AUC for the 0−120 min period did not differ between the groups.

DISCUSSION

Both the source and degree of hydrolysis of dietary protein are known to influence the absorption rate of these compounds

Table 3. Total Area under the Curve for Plasma Amino Acids (mmol·min/L) for the 0−60 min Period[a]

	soy		whey		*P* value		
	protein	hydrolysates	protein	hydrolysates	hydrolysis	source	hydrolysis × source
Ala	2.18 ± 0.90	4.68 ± 0.83	4.74 ± 0.44	7.40 ± 1.44	0.022	0.038	0.931
Arg	3.06 ± 0.35	2.00 ± 0.16	4.73 ± 0.40	3.23 ± 0.32	0.001	0.007	0.448
Asn	1.59 ± 0.22	1.38 ± 0.22	2.76 ± 0.24	1.82 ± 0.36	0.001	0.135	0.066
Asp	TRA		TRA				
Cys	0.08 ± 0.07	0.56 ± 0.14	0.24 ± 0.09	0.73 ± 0.13	0.229	0.002	0.976
Gln	5.90 ± 1.35	5.11 ± 1.76	9.54 ± 1.34	4.73 ± 1.44	0.363	0.072	0.270
Glu	0.44 ± 0.18	1.04 ± 0.33	0.66 ± 0.18	1.41 ± 0.22	0.168	0.046	0.713
Gly	1.50 ± 0.39	1.12 ± 0.54	2.98 ± 0.37	1.15 ± 0.57	0.209	0.029	0.226
His	0.62 ± 0.17	0.81 ± 0.25	1.04 ± 0.19	1.06 ± 0.19	0.152	0.626	0.690
Ile	2.37 ± 0.17	5.62 ± 0.48	3.62 ± 0.41	6.77 ± 0.58	0.005	<0.001	0.870
Leu	3.55 ± 0.36	8.16 ± 0.70	5.59 ± 0.66	14.39 ± 1.27	<0.001	<0.001	0.013
Lys	4.04 ± 0.19	7.69 ± 0.73	6.26 ± 0.37	11.55 ± 0.99	0.001	0.001	0.127
Met	0.28 ± 0.05	0.79 ± 0.13	0.58 ± 0.08	1.70 ± 0.18	0.001	<0.001	0.025
Phe	0.88 ± 0.12	0.94 ± 0.14	1.35 ± 0.16	1.54 ± 0.20	0.010	0.478	0.699
Pro	2.32 ± 0.26	4.06 ± 0.71	3.78 ± 0.32	4.90 ± 0.57	0.042	0.037	0.532
Ser	1.69 ± 0.26	2.39 ± 0.46	3.10 ± 0.35	3.45 ± 0.43	<0.001	0.352	0.398
Thr	1.65 ± 0.31	4.20 ± 0.78	3.10 ± 0.29	4.73 ± 0.83	0.019	0.036	0.212
Trp	0.51 ± 0.05	1.40 ± 0.19	0.86 ± 0.14	2.03 ± 0.16	0.009	<0.001	0.369
Tyr	0.94 ± 0.11	1.27 ± 0.29	2.04 ± 0.32	2.63 ± 0.41	0.001	0.231	0.606
Val	3.30 ± 0.33	6.69 ± 0.92	5.90 ± 0.67	9.13 ± 0.77	0.006	0.003	0.903

[a] Values are means ± SEM, *n* = 5/group. TRA, detected at trace level.

Table 4. Total Area under the Curve for Plasma Amino Acids (mmol·min/L) for the 0−120 min Period[a]

	soy		whey		*P* value		
	protein	hydrolysates	protein	hydrolysates	hydrolysis	source	hydrolysis × source
Ala	2.92 ± 2.02	4.77 ± 1.19	7.76 ± 2.57	8.23 ± 4.24	0.681	0.186	0.807
Arg	5.63 ± 0.58	6.58 ± 0.78	3.65 ± 0.35	4.32 ± 0.71	0.233	0.011	0.823
Asn	2.97 ± 0.45	3.74 ± 0.45	2.29 ± 0.46	2.11 ± 0.54	0.434	0.086	0.218
Asp	TRA		TRA				
Cys	0.06 ± 0.17	0.31 ± 0.21	1.39 ± 0.40	1.10 ± 0.32	0.964	0.002	0.456
Gln	9.86 ± 2.84	10.97 ± 4.29	10.32 ± 4.45	3.81 ± 4.41	0.567	0.404	0.423
Glu	0.77 ± 0.26	1.00 ± 0.33	1.76 ± 0.79	1.95 ± 0.40	0.615	0.156	0.954
Gly	1.92 ± 1.00	2.51 ± 0.93	0.89 ± 1.44	−1.27 ± 1.68	0.646	0.033	0.427
His	1.09 ± 0.38	1.05 ± 0.40	1.51 ± 0.62	0.91 ± 0.52	0.597	0.750	0.642
Ile	4.78 ± 0.43	5.15 ± 0.72	10.74 ± 0.85	9.27 ± 0.67	0.483	<0.001	0.251
Leu	6.77 ± 0.81	7.24 ± 1.08	16.12 ± 1.39	20.48 ± 1.59	0.118	<0.001	0.196
Lys	7.07 ± 0.29	8.50 ± 0.94	13.88 ± 1.55	16.17 ± 1.92	0.260	0.001	0.787
Met	0.37 ± 0.10	0.52 ± 0.17	1.34 ± 0.24	2.14 ± 0.34	0.096	<0.001	0.235
Phe	1.55 ± 0.27	1.58 ± 0.30	1.35 ± 0.34	1.50 ± 0.37	0.811	0.633	0.885
Pro	4.20 ± 0.62	5.37 ± 0.74	7.00 ± 1.83	5.79 ± 1.54	0.986	0.286	0.385
Ser	2.70 ± 0.56	4.01 ± 0.65	3.89 ± 0.99	3.60 ± 0.54	0.470	0.644	0.263
Thr	2.80 ± 0.67	4.22 ± 0.65	8.07 ± 1.62	6.37 ± 1.28	0.893	0.025	0.162
Trp	0.96 ± 0.14	1.14 ± 0.31	3.12 ± 0.54	3.18 ± 0.32	0.792	<0.001	0.892
Tyr	1.84 ± 0.25	3.11 ± 0.64	2.53 ± 0.59	2.96 ± 1.51	0.312	0.791	0.607
Val	6.85 ± 0.79	8.81 ± 1.30	13.40 ± 2.23	12.82 ± 1.71	0.714	0.008	0.504

[a] Values are means ± SEM, *n* = 5/group. TRA, detected at trace level.

(*1*−*3*, *7*−*12*). However, little is known of the effect in humans of protein fractionation on the rate of appearance of amino acids and bioactive peptides in the circulation and also how this absorption may be influenced by the protein source. This study demonstrated that whey protein hydrolysates ingested without carbohydrate caused a significant increase in the levels of plasma amino acids, dipeptides, and insulin compared to whey protein, soy protein, and soy protein hydrolysates.

Our study showed that whey protein induced more rapid increases in plasma amino acid levels than soy protein even though both of these proteins are classified as fast proteins. One possible explanation for this difference may relate to gastric emptying. For example, casein proteins clot at low pH, whereas whey does not, and therefore the acid environment in the stomach may cause casein to aggregate into a gel, resulting in slower release of amino acids (*1*, *4*, *5*). It is also known that the solubility of soy protein is affected by pH, temperature, and ion intensity (*39*). It is therefore possible that these differences in the digestive properties of whey and soy protein may contribute to variations in absorption rate. We also measured the molecular composition of the protein samples used in this study (**Figure 1**). The molecular size of soy protein was larger than that of whey. In addition, the molecular compositions were different between soy protein hydrolysates and whey protein hydrolysates, despite the average peptide lengths being similar. In particular, whey protein hydrolysates contained smaller molecular peptides than soy protein hydrolysates. Therefore, plasma amino acid levels may be affected not only by the digestive properties but also by molecular weight distribution.

Our study also demonstrated that plasma amino acid levels increased rapidly after ingestion of both soy and whey protein

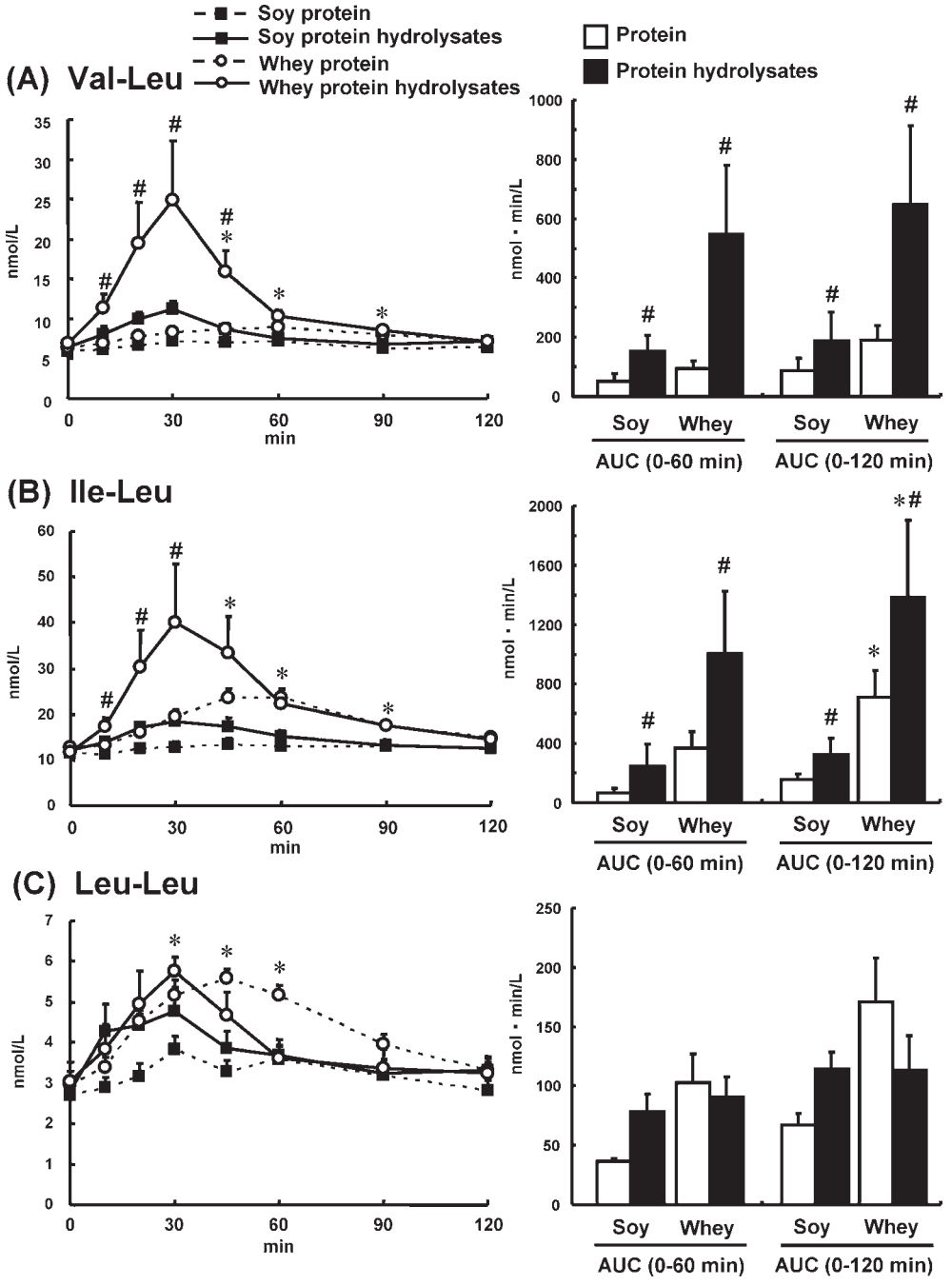

Figure 3. Plasma concentrations of (**A**) Val-Leu, (**B**) Ile-Leu, and (**C**) Leu-Leu, the main components of BCAA-containing bioactive peptides. The left-hand graphs show the plasma concentrations of these compounds over the 120 min study period. The right-hand graphs show the area under the curve (AUC) for the 0−60 and 0−120 min periods. Values are expressed as means ± SEM, $n = 5$/group. #, $P < 0.05$ significant difference between nonhydrolyzed protein and protein hydrolysates; ∗, $P < 0.05$ significant difference between dietary protein source.

hydrolysates compared to nonhydrolyzed protein. Only a few studies in humans have demonstrated this more rapid increase in plasma amino acid levels after consumption of whey protein hydrolysates compared to intact whey protein in human subjects. In previous papers, Calbet and MacLean (*31*) showed that whey protein hydrolysates elicited a faster increase in venous plasma amino acids than did a milk solution. They also demonstrated that plasma IAA and BCAA concentrations increased rapidly after ingestion of casein hydrolysates compared to casein, although they showed no difference between whey protein and whey protein hydrolysates (*5*). Dietary protein hydrolysates are a complex mixture of peptides of different chain lengths and free amino acids. The protein hydrolysates used in this study contained mainly low molecular weight peptides (average peptide length = 3.01). A number of studies in humans have shown that the same nitrogen load is absorbed more rapidly when delivered as di- and tripeptides rather than as whole protein or free amino acids (*7*−*12*). These findings therefore indicate that differences in the peptide chain length profile of dietary protein hydrolysates may affect plasma amino acid responses.

Power et al. (*40*) demonstrated that plasma BCAA levels were similar with whey protein and whey protein hydrolysates even though they used short-chain whey protein hydrolysates similar to those used in our study. One possible reason for this discrepancy may be a difference in whey protein type. Whey protein is classified into mainly whey protein concentrate (WPC)

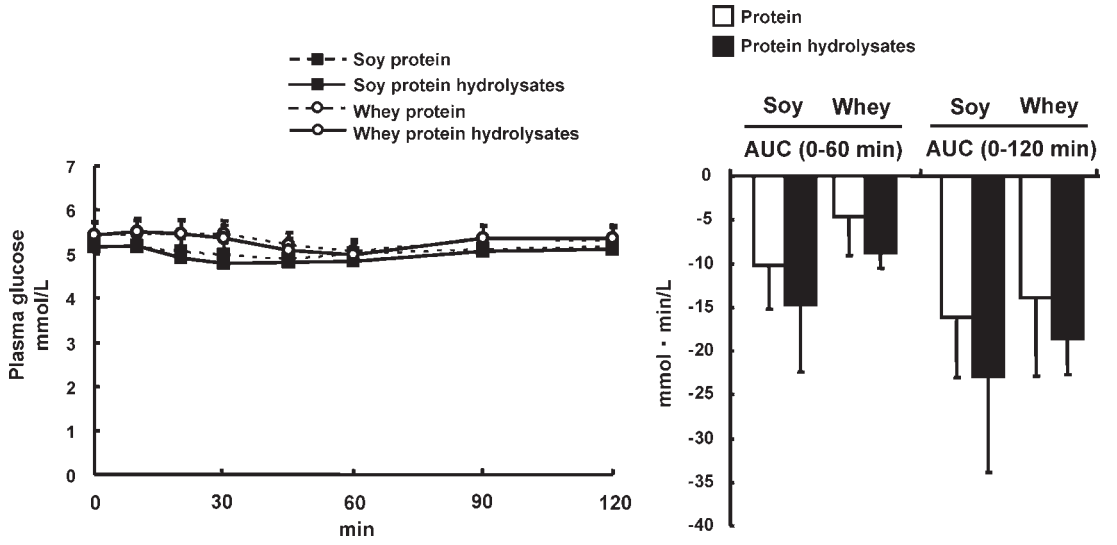

Figure 4. Plasma glucose concentration. The left-hand graph shows the plasma concentration of insulin over the 120 min study period. The right-hand graph shows the area under the curve (AUC) for the 0−60 and 0−120 min periods. Values are expressed as means ± SEM, n = 5/group.

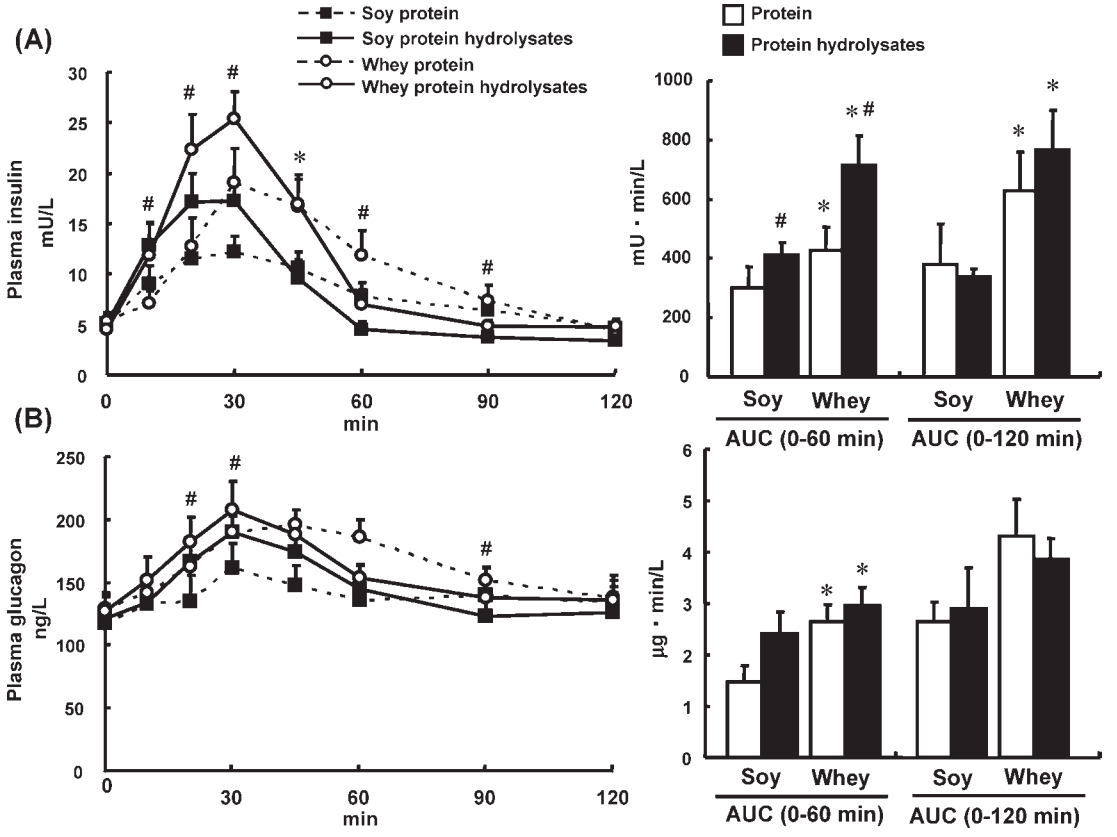

Figure 5. Plasma (**A**) insulin and (**B**) glucagon concentrations. The left-hand graphs show the plasma concentration of insulin over the 120 min study period. The right-hand graphs show the area under the curve (AUC) for the 0−60 and 0−120 min periods. Values are expressed as means ± SEM, n = 5/group. #, $P < 0.05$ significant difference between nonhydrolyzed protein and protein hydrolysates; ∗, $P < 0.05$ significant difference between dietary protein source.

or whey protein isolates (WPI) produced by different processing methods. It is known that the amounts of carbohydrate, fat, and protein and also the compositions of the milk protein fraction are different in WPI and WPC, with the latter containing higher amounts of large molecular proteins such as bovine serum albumin (*41*). In this study, we used WPC as the protein source, whereas Power et al. used WPI. Therefore, the variable results observed in these studies may be attributable to the different sources of whey protein used in the investigations.

There is increasing interest in the use of bioactive peptides in the development of functional food products. A small number of nutritional studies have reported that dietary bioactive peptides are absorbed as intact forms and then circulate in the blood (*17*). Recently, we identified the BCAA-containing dipeptides Ile-Val, Leu-Val, Val-Leu, Ile-Ile, Leu-Ile, Ile-Leu, and Leu-Leu in whey

protein hydrolysates. These peptides were shown to stimulate glucose uptake in skeletal muscle in vitro (*18*). The current study showed these BCAA-containing dipeptides were detectable in human plasma after ingestion of whey protein hydrolysates. Furthermore, the plasma levels of Val-Leu and Ile-Leu, in particular, increased significantly after ingestion of protein hydrolysates compared to nonhydrolyzed protein. Dietary protein hydrolysates are generally produced from purified protein sources by heating with acid or, preferably, by incubation with proteolytic enzymes such as peptidase from *Aspergillus oryzae*, followed by purification procedures. It is therefore possible that bioactive peptide-rich protein hydrolysates produced by industrial enzymes, which cleave specific peptide bonds, may increase the bioavailability of bioactive peptides compared to protein digested by gastrointestinal enzymes. In this study we observed that the plasma concentrations of Val-Leu and Ile-Leu levels were higher in whey protein hydrolysates than in soy protein hydrolysates. β-Lactoglobulin, the main component of whey protein, has numerous amino acid sequences of BCAA-containing dipeptides compared to other protein sources (*18*). Furthermore, we demonstrated the BCAA-containing dipeptide content in whey protein hydrolysates was shown to be higher than that in soy protein hydrolysates (**Table 1**). Therefore, both the number of amino acid sequences of target peptides in dietary protein and the target peptide contents in protein hydrolysates may also be important factors determining the bioavailability of bioactive peptides.

Our results also showed that plasma Val-Leu and Ile-Leu kinetics were different from plasma Leu-Leu kinetics. It is possibile these results may be related to the stability of the dipeptides. It is well-known that aminopeptidases catalyze the cleavage of amino acids from the amino terminal of protein or peptide substrates (*42*). Leucine aminopeptidases (LAPs), which are widely distributed in blood and tissues, are metallopeptidases that cleave N-terminal leucine residues. Thus, N-terminal leucine dipeptides may be susceptible to aminopeptidases, such as LAPs, compared to N-terminal valine and isoleucine dipeptides.

It is also well-known that milk proteins have insulinotropic properties, with the whey fraction being a more efficient insulin secretagogue than casein (*25*, *43*). In contrast, a small number of nutritional studies have reported comparisons of the insulinotropic effect of soy and whey proteins, which are both classified as fast proteins. Classens et al. (*44*) showed that when compared with carbohydrate ingestion only, the insulin response increased significantly after co-ingestion of whey protein hydrolysates and carbohydrate, but not after co-ingestion of soy protein hydrolysates and carbohydrate. Veldhorst et al. (*45*) demonstrated that a whey protein meal triggered the strongest responses in insulin concentrations compared with either a casein or soy meal. Almost all research in this area has compared the results with the insulinotropic effect of dietary protein from different sources coingested with carbohydrate. The current study also demonstrated clearly that ingestion of whey protein without carbohydrate significantly stimulated insulin release compared to soy protein.

The insulinotropic effect of whey protein may be related to the postprandial level and composition of plasma amino acids. Several in vitro studies using incubated β pancreatic cells also showed that arginine, isoleucine, phenylalanine, and leucine in combination with glutamine had a strong insulinotropic effect (*16*, *46*). Nilsson et al. (*25*) reported a close relationship between the insulin response and increase in plasma amino acid levels in humans. This relationship was especially apparent for leucine, isoleucine, valine, lysine, and threonine, which are all known to stimulate insulin secretion following the ingestion of whey protein (*24*, *27*). They also demonstrated that the insulinotropic responses after a glucose drink containing these five amino acids and after a glucose-equivalent drink containing whey proteins did not differ significantly (*47*). Our study provides further evidence that whey protein hydrolysates cause marked increases in the plasma concentrations of some amino acids, especially isoleucine, leucine, lysine, threonine, and valine. We also found a significant, positive correlation between the levels of plasma insulin and these amino acids, such as isoleucine ($P < 0.001$, $r = 0.684$), leucine ($P < 0.001$, $r = 0.719$), lysine ($P < 0.001$, $r = 0.691$), threonine ($P < 0.001$, $r = 0.491$), and valine ($P < 0.001$, $r = 0.616$). Thus, the relationships we observed following the ingestion of protein only agreed with those measured after co-ingestion of protein and carbohydrate.

Furthermore, we demonstrated that the plasma insulin response for the 0−60 min period was higher for protein hydrolysates than for whole protein, whereas the responses for the 0−120 min period were not different between the two groups. Similar responses were observed for amino acids. These findings therefore indicate that postprandial plasma amino acid composition and concentration, in particular, isoleucine, leucine, lysine, threonine, and valine, are important factors that determine the insulinotropic properties of dietary proteins.

This study showed that plasma glucose levels were maintained within the normal range and were not different between treatments, even though ingestion of whey protein hydrolysates caused marked increases in plasma insulin concentration. Glucagon plays a major role in counteracting insulin-induced hypoglycemia (*48*). Dietary proteins are also known to induce glucagon secretion, a response that is mediated by the action of amino acids in the blood on pancreatic α-cells (*26*, *31*, *44*, *49*). However, in the current study we demonstrated that plasma glucagon levels for the 0−120 period were not different between the groups. Classens et al. (*44*) demonstrated that the magnitude of the glucagon response varies more with the protein type than the magnitude of the insulin response dose, which was influenced more by carbohydrate co-ingestion. One possible reason for this discrepancy may be due to the protein dosage or co-ingestion of carbohydrate.

Taken together, the findings of this study demonstrate that the source and degree of hydrolysis of dietary protein influence plasma amino acid and dipeptide concentrations. In particular, the ingestion of whey protein hydrolysates, independent of carbohydrate, caused a significant increase in the responses of plasma amino acids, dipeptides, and insulin compared to whey protein, soy protein, and soy protein hydrolysates.

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