Refining in vitro digestibility assays: Fractionation of digestible and indigestible peptides[1]

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ABSTRACT: Typically, in vitro methods used for estimating the amount of ileal digestible AA do not exhaustively digest samples, and arbitrary methods for separating digestible from indigestible protein are used. This may lead to over- or underestimation of digestibility coefficients. A method that exhaustively digests proteins using pepsin and pancreatin was developed, and the first objective of this research was to confirm that exhaustive digestion was indeed appropriate and to determine the fractionation method for separating digestible from indigestible proteins. For this, three homoarginine-labeled animal proteins were prepared. Samples were subsequently digested in vivo and in vitro to determine which fraction should be considered indigestible, and in vitro followed by in vivo to determine whether the extent of digestion in vivo was improved by predigestion. In vivo, soluble but unabsorbed peptides were smaller than 1 kDa, suggesting that the size of soluble peptides is not what prevents their absorption. Thus, all in vitro-soluble proteins should be considered digestible. In vitro, 88 ± 3% of the soluble peptides were smaller than 1 kDa, with the remainder between 1 and 5 kDa, suggesting that in vitro digestion is less complete. Predigested samples were digested in vivo to the same size distribution as the nonpredigested samples. The second objective was to test whether in vitro digestibility assays based on these principles equaled in vivo digestibility. For this, digestibility data for 25 animal proteins were compared. Results showed a lack of correlation between lysine digestibility coefficients; however, across samples, the extent of digestion did not differ for lysine ($P = 0.71$), threonine ($P = 0.26$), methionine ($P = 0.18$), or valine ($P = 0.66$), whereas in vitro digestibility coefficients were lower for (the less water-soluble) histidine ($P = 0.05$), isoleucine ($P < 0.01$), leucine ($P < 0.01$), and phenylalanine ($P = 0.05$). In conclusion, in vitro digestibility assays should exhaustively digest proteins to mimic in vivo digestibility. All in vitro-soluble peptides could be considered digestible, because in vivo, no large soluble peptides were observed whose size prevented them from being absorbed. However, an in vitro assay based on these principles lacked precision for highly water-soluble AA, and underestimated digestibility for other AA. Better solubilization of the digesta and more replicates may improve the in vitro assay further.

Key Words: Amino Acid Digestibility, Animal Protein, In Vitro, In Vivo

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Introduction

Ileal AA digestibility is a well-accepted indicator of protein quality (Darragh and Hodgkinson, 2000). However, measuring digestibility is costly and time consuming. Due to this shortcoming, in vitro techniques have been explored (Boisen and Fernández, 1995; Boisen and Moughan, 1996). These methods typically use pepsin and pancreatic proteases to mimic digestive functions in vivo. Simulation of the in vivo absorption process is applied based on the solubility or molecular sizes of digested peptides. The underlying assumption is that peptides that are insoluble or greater than a critical size (molecular weight cut-off) are not absorbed in vivo.

The reported digestibility coefficients, however, are typically lower by as much as 40% than those measured in vivo (Brule and Savoie, 1988; Savoie et al., 1989). Incomplete digestion resulting from short incubation time and/or insufficient enzyme use might be reasons for the underestimation. Choice of the molecular weight cut-off for separating digestible from indigestible peptides (1 kDa in these cases) might also affect the estimation, as too small a molecular weight cut-off would allow only very small peptides to be separated as digestible.

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Table 1. Amino acid composition of three guanidinated animal proteins used in the in vivo and in vitro assays (%, as-fed basis)

Amino acid	Animal protein sample		
	A	B	C
Arginine	3.65	3.13	3.94
Cysteine	1.16	0.38	1.27
Histidine	0.81	1.11	1.28
Isoleucine	1.71	1.67	2.05
Leucine	3.41	3.20	4.19
Lysine	0.47	0.90	0.66
Homoarginine[a]	1.86	1.69	2.50
Methionine	0.63	0.77	0.84
Phenylalanine	1.94	1.76	2.33
Threonine	1.93	1.70	2.21
Valine	2.68	2.30	3.14

[a]Homoarginine, obtained through guanidination of the lysine residues. Conversions of lysine to homoarginine, calculated as [homoarginine/(homoarginine + lysine) × 100] were 80, 65, and 79% for samples A, B, and C, respectively.

To quantify AA digestibility, a new method has been developed that exhaustively digests proteins based on the hypothesis that the digestive capacity of the animal is not limiting the extent of digestion. Enzyme use in this method is minimized, and potential contamination from digested enzymes is corrected for (Qiao et al., 2002). Separation of indigestible and digestible proteins, however, remains contentious.

The objectives of this research were 1) to study the size distribution of soluble peptides in vitro and in vivo so as to define a fractionation method for in vitro protein digestibility assays and to confirm whether proteins are exhaustively digested in vivo, and 2) to validate the resulting in vitro method by comparing in vitro and in vivo digestibility.

Materials and Methods

Materials

Sodium citrate, citric acid, sodium phosphate dibasic, sodium phosphate monobasic, methanol (HPLC grade), and trifluoroacetic acid (HPLC grade) were obtained from Fisher Scientific (Atlanta, GA). Peptides (HPLC grade, Table 1) used for size exclusion chromatography were obtained from Sigma (St. Louis, MO). *O*-Methylisourea was purchased from SKW Chemicals (Marietta, GA). Animal protein samples were obtained from the Fats and Protein Research Foundation (Bloomington, IL). These included 25 samples that had been assayed in vivo for digestible AA using the cecectomized cockerel assay and a protein-free diet for determining basal endogenous losses (Parsons, 1986).

Guanidination of Animal Protein

To determine the extent of digestion of proteins in animal meals, three meat and bone meal samples were labeled with homoarginine (Table 1). Homoarginine is not a naturally occurring AA, but because it acts like lysine, its digestion can be used as a marker for feedstuff protein digestion (Nyachoti et al., 1997). Guanidination of these samples was performed according to published methods (Maga, 1981; Siriwan et al., 1994; Nyachoti et al., 1997). In brief, 15 L of 0.5 mol/L *O*-methylisourea in 1 mol/L of NaOH solution was added to 6 kg of animal protein (for an *O*-methylisourea to protein ratio of 0.43, wt/wt). The resulting broth was stirred, and the pH was maintained at 10.5 while the reaction was allowed to take place at room temperature. After 4 d, the animal protein mixture was neutralized with 6 mol/L of HCl, and then washed four times to remove remaining *O*-methylisourea. The guanidinated animal protein was subsequently freeze-dried, and the homoarginine content in each animal protein sample was determined at the Experimental Station Chemical Laboratories at the University of Missouri (Columbia) using AOAC methodology (AOAC, 1995) to verify the extent of guanidination. The AA compositions of the guanidinated animal protein samples are presented in Table 1.

In Vitro Digestibility

The in vitro assay that was used in this study was a two-stage method that was developed to maximize the hydrolysis of the animal protein peptide bonds with minimal enzyme usage (Qiao, 2001). In brief, in Stage 1, pepsin (120 U/mL, or 0.25% enzyme protein relative to substrate protein) was used to digest the substrate proteins (12.5 mg/mL) in citrate buffer solution (pH 2) for 24 h. For Stage 2, phosphate buffer solution for a final pH of 8 and trypsin-enriched pancreatin (activity equivalent to at least three U.S. Pharmacopeia/mL, or 7.5% enzyme protein relative to substrate protein, final substrate concentration 5 mg/mL) were added and the digestion was continued for an additional 96 h. The length of these incubation times correspond to the time needed to loose over 95% of the activity of the enzymes in order to maximize their efficacy, thus allowing for minimal enzyme usage. Incubations were carried out at 38°C under continuous agitation.

For the guanidinated animal proteins, the digestion was carried out in large containers fitted with two aquarium heaters to prepare samples for the feeding trial, and in triplicate using 50-mL centrifuge tubes for measurement of digestibility. For the 25 animal meal samples, the digestion was carried out in triplicate in 50-mL centrifuge tubes.

Ileal Digestibility

This study was approved by the North Carolina State University Institutional Animal Care and Use Committee.

To determine whether the extent of in vivo digestion is improved by predigesting the sample, guanidinated

Table 2. Composition (%, as-fed basis) of experimental diets fed to ileum-cannulated pigs

Ingredient	Content
Cornstarch	40.67
Sucrose	20.70
Corn oil	2.07
Salt	0.35
Dicalcium phosphate	3.11
Limestone	0.52
Mineral and vitamin mix[a]	0.35
Chromic oxide	0.55
Animal protein[b]	31.68

[a]Provided per kilogram of diet: 2,000 IU of vitamin A; 300 IU of vitamin D_3; 20 IU of vitamin E; 1.0 mg of vitamin K (menadione); 4 mg of thiamine; 15 mg of niacin; 4 mg of riboflavin; 12 mg of pantothenic acid; 15 μg of vitamin B_{12}; 2 mg of pyridoxine; 0.1 mg of d-biotin; 0.5 mg of folic acid; 0.6 g of choline; 90 mg of Fe (ferrous sulfate); 5 mg of Mn (manganese oxide); 8 mg of Cu (copper sulfate); 0.20 mg of I (potassium iodate); 0.21 mg of Se (sodium selenite); and 90 mg of Zn (zinc sulfate).

[b]Animal protein: Three animal proteins, A, B, and C, were used. Regular nonguanidinated animal proteins were fed to pigs on adaptation days, whereas guanidinated animal proteins were fed to pigs on day of collection.

meat and bone meal was tested for ileal digestibility as is, or after predigestion in vitro. Failure to show improved digestibility with predigested material would suggest that in vivo digestion is exhaustive and that in vitro assays should digest to exhaustion as well. To estimate the size distribution of soluble but unabsorbed peptides, ileal and in vitro digestibility assays were performed with guanidinated animal protein samples. In vivo-soluble but undigested proteins and in vitro digests were subsequently size separated for determination of the faith of homoarginine, the assumption being that besides insoluble proteins, large soluble peptides of a size to be quantified were indigestible.

The design of this trial was a 6 × 6 Latin square with six diets (three guanidinated animal protein samples, as is or predigested in vitro), six periods (each of one week), and six castrated pigs (53 ± 1.3 kg BW) fitted with ileal T-cannulae. Animal protein was the sole protein source in the diets (Table 2). Each period consisted of a 6-d adaptation period and a 1-d collection period. During the adaptation period, pigs were fed diets composed of the corresponding nonguanidinated and undigested animal protein twice daily (0600 and 1600) at approximately 45 g/kg$^{0.75}$ BW per meal (adjusted weekly). On collection days, pigs were fed guanidinated animal meal (fortified with chromic oxide as a marker for the indigestible fraction) for the morning feeding, whereas no feed was supplied in the evening. Collection of ileal juices started at the morning feeding (0600) and continued for 19 h. This collection period was sufficient to collect ileal content corresponding to the test diet (as judged by chromic collection). The ileal juices were centrifuged at 1,000 × g for 30 min to separate soluble proteins from insoluble ones. These fractions were kept frozen at −20°C until analysis for molecular weights of peptides by size exclusion chromatography and AA contents by HPLC.

All AA and chromium analyses were carried out at the Experimental Station Chemical Laboratories using AOAC-approved methodology (AOAC, 1995). Amino acid recovery exceeded 96.4%, (T. Mawhinney, University of Missouri, personal communication), and no corrections were made for incomplete recovery resulting from hydrolysis. The ileal AA digestibilities of the guanidinated animal protein samples in the solid feeds were calculated according to van Kempen et al. (2002). These digestibility figures are apparent for all AA except homoarginine, for which real digestibility is calculated due to the absence of homoarginine in endogenous secretions.

$$\text{Digestibility, \%} = 100 - [(M_d \times AA_I)/(AA_d \times M_I)] \times 100$$

where M_d = chromium concentration in the diet (mg/kg), AA_I = AA concentration in ileal digesta (g/kg), AA_d = AA concentration in the diet (g/kg), and M_I = chromium concentration in the ileal digesta (mg/kg).

The ileal AA digestibility coefficients were analyzed with the PROC GLM procedure of SAS (Version 7, SAS Inst., Inc., Cary, NC). To compare treatment effects (in vitro vs. in vivo) on the digestibility coefficients of the three guanidinated meat and bone meals samples, the in vitro and in vivo AA digestibility coefficients were analyzed with the PROC MIXED procedure of SAS using the model:

$$Y_{ijk} = \mu + S_i + D_j + (DS)_{ij} + A(D)_{j(i)} + P(D)_{k(i)} + \varepsilon_{ijk}$$

where Y_{ijk} = response variable (AA digestibility coefficient) for the ith sample (S_i, i = 1, 2, 3), jth digestion treatment (D_j, digestion treatment = in vivo, in vitro), and kth period (k = 6 levels [six animals, six periods, missing values were treated as such] for in vivo trial, k = 1 level [three test tubes, one period] for in vitro trial), S_i = MBM sample effect, D_j = digestion effect, $(DS)_{ij}$ = interaction between sample and digestion, $A(D)_{j(i)}$ = animal (test tube) effect within the digestion treatment, $P(D)_{k(i)}$ = period effect within the digestion treatment, μ = overall mean, ε_{ijk} = residual error with mean of 0 and variance of σ^2. The effects $A(D)_{j(i)}$ and $P(D)_{k(i)}$ were treated as random effects by using the RANDOM statement.

Size Exclusion Chromatography

Size separation of ileal and in vitro digesta was conducted with a Tosohaas 2000SWXL column (Montgomeryville, PA) suitable for separation of 500- to 100,000-Da polypeptides (Swergold and Rubin, 1983). The column (7.8 mm i.d. × 30 cm in length, silica-based packing, particle size = 5 μm, and pore size = 125 Å) was fitted with a guard column (6 mm i.d. × 4.0 cm in length, silica-based packing, particle size of 7 μm). The HPLC system consisted of a model 600 Controller, a model

Table 3. Size of peptides used to calibrate the size exclusion column[a]

Item	Molecular weight, Da
Leucylglycine	176
Homoarginine·HCl	225
Gly-Gly-Phe-Phe	455
Casein fragment	913
Bombesin	1,620
Diazepam binding inhibitor (DBI, fragment 51 to 70)	2,150
Insulin	5,730
Ubiquitin	8,565
Cytochrome C	12,327
Lysozyme	14,400

[a]Peptides were purchased from Sigma Chemical Co. (St. Louis, MO).

717 autosampler, and a model 996 photodiode array detector (Waters Inc., Milford, MA). The mobile phase was an aqueous solution of 35% methanol and 0.1% trifluoroacetic acid, selected based on solubility of different peptides (Swergold and Rubin, 1983; Irvine and Shaw, 1986; Vijayalakshmi et al., 1986).

Ten peptides of known molecular weight were used for calibration of the column (Table 3). These peptides were dissolved in the mobile phase (0.1%, wt/vol) and filtered through a 0.1-μm Millipore filter (Sigma) before they were individually run on the column (material remaining on the filter was considered insoluble). Mobile phase was degassed with an in-line degasser (Waters Inc.). The injection volume was 20 μL, and the flow rate was 0.51 mL/min. The detection wavelength was set at 214 nm. Retention times of these peptides were regressed against their molecular weight using linear regression (SAS), and the obtained regression equation was used for predicting the molecular weight of size-fractionated digesta/digests.

For size separation of the ileal indigestible peptides, ileal juices were first centrifuged at 1,000 × *g* for 10 min. The supernatants were then dried at 35°C using an evaporator (Centrivap Console, Labconco, Kansas City, MO). Dried supernatants were pooled on an equal weight basis according to dietary treatment and then resuspended in mobile phase (with magnetic stirring) at 0.2 g/mL. The resuspensions were filtered with No. 4 filter paper (Whatman Int., Ltd., Maidstone, U.K.) and washed with an equal volume of mobile phase. The final concentration of the re-suspension was around 0.1 g/mL. The filtrates were further filtered with a 0.45-μm Millipore filter (Sigma) before loading onto the column.

For size separation of in vitro peptides, in vitro digests of the three guanidinated animal proteins also underwent centrifugation, drying, resuspension, and filtration as described above for the ileal samples. Because the in vitro digests contained a much higher concentration of homoarginine than ileal juice, the dried supernatant was resuspended in mobile phase at 0.05 g/mL.

In vitro and ileal samples were loaded on the column and chromatographed under conditions as described for the calibration of the column. The eluent was collected with a fraction collector (model FC-80K, Gilson Medical Electronics Inc.; Middleton, WI) at 1-min intervals from time 0 to 36 min after loading. To ensure there was sufficient homoarginine for AA analysis, each ileal juice sample was run at least 12 times, and each in vitro digesta sample was run at least seven times. The eluents were pooled according to sample and elution time and analyzed for homoarginine contents by the Experimental Station Chemical Laboratories using AOAC methodology (AOAC, 1995).

Comparison of In Vivo and In Vitro Digestibility

Twenty-five animal meal samples with known standardized digestibility were obtained. These samples had been evaluated for digestibility using cecectomized cockerels (n = 5 per sample) and consisted of (as-fed basis) 19 meat and bone meals (CP = 44.6 ± 4.1%) and six feather meals (CP = 77.2 ± 3.0%).

These samples were digested in vitro as described above. In vitro digestibility data were corrected for enzyme added which was hydrolyzed into peptides considered digestible (enzyme contamination). This contamination was determined by performing the in vitro assay without added substrate (Qiao et al, 2002).

Soluble peptides, obtained after centrifugation at 15,000 × *g* for 20 min were considered as digestible. Amino acids in this fraction were analyzed at the Experimental Station Chemical Laboratories using AOAC methodology (AOAC, 1995). The in vitro AA digestibility was calculated with the following formula:

$$Digestibility = \frac{AA_{\text{protein in supernatant}} - AA_{\text{enzyme in supernatant}}}{AA_{\text{animal protein}}}$$

The in vitro AA digestibility coefficients of the 25 reference samples were compared with the known in vivo AA digestibility coefficient. The null hypothesis "in vitro – in vivo = 0" was tested by a two-tailed paired *t*-test using the PROC TTEST procedure.

Results

Size Fractionation of Peptides

In the size-fractionation study, a high correlation between $\log_{10}$ molecular weight (Da) and elution time was found ($\log_{10}$ molecular weight = –0.223 (± 0.016) × time (min) + 7.03 (± 0.28), R^2 = 0.96, Figure 1). For small peptides (below the specified lower size separation limit of the column), the elution time was affected by their size and their electrical charges (ion exchange), in line with findings from Irvine and Shaw (1986). Therefore, elution times longer than 20 min were considered as 20 min (383 Da).

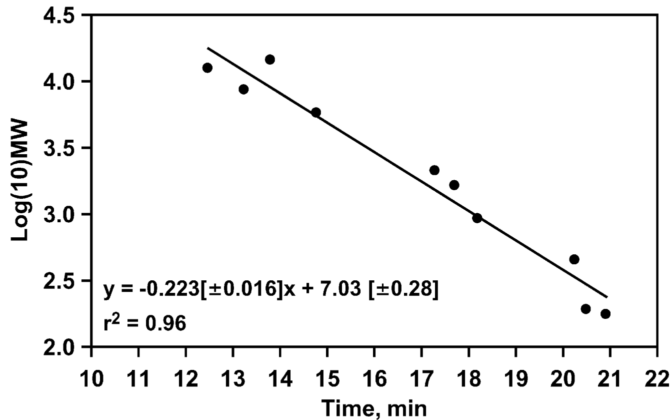

Figure 1. Size exclusion chromatography of soluble peptides of known molecular weight (MW).

Digestibility of Homoarginine In Vivo and In Vitro

Diet refusal was observed for three animals upon switching from nonguanidinated to guanidinated animal meals. Data from these animals was excluded from the analyses (Table 4).

On average, 75% of the lysine in the meat and bone meal samples was converted to homoarginine (Table 1). Homoarginine digestibility ranged from 79 to 90% in vivo (Table 4), and was significantly different between samples. Because homoarginine is not a naturally occurring AA, these values were considered to be the real extent of digestion of the homoarginine in the samples (real digestibility). Approximately half the indigestible homoarginine was recovered in the insoluble fraction (42 to 51%); the other half was in soluble peptides. These soluble peptides were all less than 1 kDa, with nearly all of this being 383 Da or less (98.5 ± 1.3%, Figure 2).

Digestibility of lysine ranged from 9 to 23%. These values are apparent digestibilities, and as lysine in these samples was artificially lowered, they were very strongly influenced by endogenous losses. Presuming that lysine and homoarginine had the same real digestibility, it can be calculated that the equivalent of 13 to 20% of the feed (lysine + homoarginine) was recovered in endogenous losses.

In vitro, 71 to 80% of the homoarginine was soluble after digestion. Across samples, these values were lower ($P < 0.01$) than the in vivo digestibility. On average, 88 ± 3.1% of the soluble homoarginine was found in fractions of molecular weight less than 1 kDa, with the remainder in fractions between 1 and 5 kDa. Although the column could elute proteins up to 100 kDa, and could quantify soluble peptides up to 15 kDa, no peptides larger than 5 kDa that contained homoarginine were found.

In vitro apparent lysine digestibilities were numerically lower than the real homoarginine digestibilities due to contamination by digestion enzyme lysine. Presuming that lysine and homoarginine had the same real digestibility, it can be calculated that the equivalent of 1% of the feed (lysine + homoarginine) was digested enzyme lysine fractionated as digestible. This matches expectations based on running digestibility assays without added substrate (Qiao, 2001).

Samples that were predigested in vitro and subsequently digested in vivo had a size distribution of the soluble fraction comparable to the in vivo digested samples, with 99.8 ± 0.2% of the soluble homoarginine observed in the fractions of less than 1 kDa. Because these diets were fed in liquid form, chromic oxide settled out

Table 4. Apparent ileal and in vitro digestibility (%) of amino acids in three guanidinated animal proteins by pigs

	Apparent ileal digestibility				In vitro digestibility					Main treatment effect
	Sample				Sample					
Amino acid	A (n = 5)	B (n = 4)	C (n = 6)	Mean (n = 15)	A (n = 3)	B (n = 3)	C (n = 3)	Mean (n = 9)	SD[a]	Variation, *P*-value
His[b]	79.6	72.2	79.7	77.6	69.1	75.1	68.9	71.0	4.9	<0.01
Homoarginine[b]	89.5	79.4	85.7	85.3	70.9	79.7	77.9	76.1	3.2	<0.01
Ile	81.1	72.1	75.8	76.6	78.2	81.7	81.5	80.5	5.0	0.07
Leu	81.1	74.8	79.9	78.9	76.3	79.4	76.0	77.2	4.8	0.45
Lys	22.6	21.8	9.2	17.0	61.9	78.7	72.1	70.9	17.4	<0.01
Met	87.7	81.8	87.8	86.2	70.4	79.4	72.7	74.2	4.3	<0.01
Phe[b]	81.6	68.6	87.8	80.6	75.5	75.6	77.1	76.1	4.4	0.06
Thr	70.7	64.7	64.3	66.6	76.8	75.4	71.5	74.6	6.4	0.01
Val	80.1	72.5	78.5	77.4	82.5	83.2	81.5	82.4	5.4	0.04

[a]Pooled standard deviation between treatments, %.
[b]Interaction between treatments and samples ($P < 0.05$).
[c]Sample difference ($P < 0.05$) in apparent ileal amino acid digestibility.

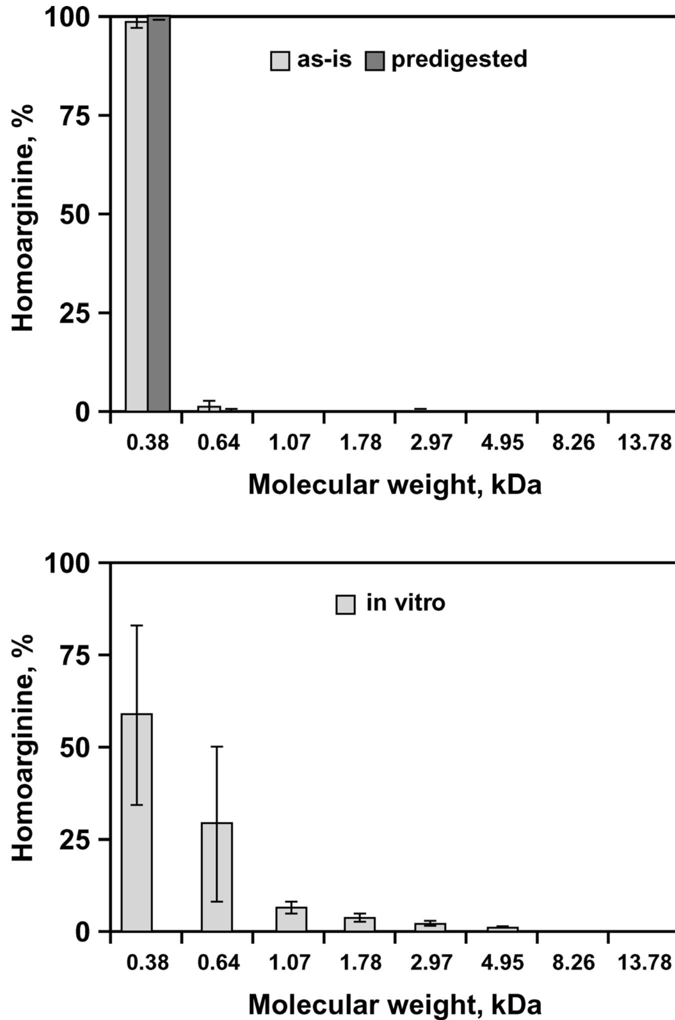

Figure 2. Size distribution of soluble, homoarginine-containing peptides (±SEM) observed at the end of the ileum (top) or after in vitro digestion (bottom). For the in vivo assay, animals were fed guanidinated meat and bone meal as the only protein source, either as is (n = 15) or predigested in vitro (n = 18).

in the feeder, thus preventing the calculation of digestibility coefficients for the predigested diets.

In Vitro and In Vivo Digestibility for Regular Samples

The 25 samples used for validating the in vitro digestibility assay covered a wide range of standardized digestibilities for lysine (from 54 to 91%) and for methionine (from 53 to 93%) (Table 5). Across samples, the mean in vivo and vitro digestibility coefficients were not different for lysine ($P = 0.71$), methionine ($P = 0.18$), threonine ($P = 0.26$), and valine ($P = 0.66$). However, in vivo digestibility was higher than the in vitro digestibility for histidine ($P = 0.05$), isoleucine ($P < 0.01$), leucine ($P < 0.01$), or phenylalanine ($P = 0.05$). Also, no significant correlations between in vitro and in vivo digestibility coefficients were observed.

Discussion

Simulation of in vivo digestion and absorption is a complex process. With modern in vitro techniques, conditions such as use of pepsin and pancreatic proteases, pH, enzyme:substrate ratios, and incubation time can be controlled, and maximal digestion of the protein substrate by pepsin and pancreatic proteases can be realized. It is very difficult in practice, however, to introduce brush border membrane (which has amino-peptidases and transporters) into the in vitro system because functioning of the brush border membrane requires living intestinal cells.

The brush border membrane carries out two essential functions: digestion and absorption. Apical microvillus-bound amino-peptidases further digest peptide bonds remaining after digestion by pepsin and pancreatin (Kidder and Manners, 1978; Sangild et al., 1991; Matsumoto et al., 1995), and peptide transporters absorb AA and short peptides (Silk et al., 1985; Webb, 1986). In essence, an important difference between in vitro and in vivo is that the in vitro system lacks the membrane-associated functions present in vivo.

The current study suggests that peptides digested maximally in vitro are broken down further in vivo. The size distribution of the in vitro peptides ranged up to 5 kDa, whereas the ileal peptides from feeding of the in vitro digesta to pigs were smaller than 1 kDa. The in vitro peptides ranging from 1 to 5 kDa (equivalent to approximately 8 to 40 AA residues) were thus removed in vivo (Figure 2). Contemporary knowledge of AA and peptides absorption suggests it is unlikely that the intestinal peptide transporters directly take up peptides from 1 to 5 kDa (Silk et al., 1985; Webb, 1986). Thus, if the in vitro digesta fed to the pigs was truly maximally digested by pepsin and pancreatic proteases, then it is logical to assume that it was the amino-peptidases that further hydrolyzed these soluble peptides. Another possibility was that the in vitro digestion was not complete due to feedback inhibition of the digestive enzymes, preventing further hydrolysis of peptides.

Our original assumption was that although the intestinal peptidases aided in digesting small soluble peptides, it was possible to correct for their absence in vitro by selecting an upper molecular size limit for peptides (e.g., 1 kDa), above which size, the intestinal epithelium had little action on the digestion of these peptides. However, in vivo, no soluble peptides of a size that prevented them from being digested were observed. In vitro, such peptides were observed, but those peptides were digestible in vivo.

In contrast to expectations, all of the in vivo-soluble but unabsorbed peptides were smaller than 1 kDa, mainly free AA and dipeptides. This fraction comprised approximately half the unabsorbed ileal peptides. This means that a significant amount of protein containing homoarginine was digested into small peptides but escaped absorption (indicative of limit peptides). This might be a natural yet poorly documented physiological

Table 5. Summary of the in vivo and in vitro amino acid digestibility coefficients (%) and comparison between in vivo and in vitro amino acid digestibility for 25 animal meal samples

	Ileal digestibility[a]			In vitro digestibility[b]			In vivo − in vitro		
Amino acid	Mean	Min	Max	Mean	Min	Max	Mean	SEM	*P*-values
His	76.1	60.2	88.6	70.8	58.9	95.7	7.9	3.0	0.05
Ile	84.5	76.6	91.6	74.8	60.1	90.0	8.6	2.0	<0.01
Leu	84.8	75.8	92.1	76.2	59.6	92.6	7.1	2.2	<0.01
Lys	78.7	54.0	90.5	76.7	65.1	90.4	1.4	2.4	0.71
Met	80.6	53.0	92.6	74.9	49.5	97.1	3.0	4.2	0.18
Phe	87.4	27.3	98.7	81.6	63.3	96.6	9.7	3.4	0.05
Thr	79.2	67.6	89.2	72.3	48.8	99.3	4.3	2.6	0.26
Val	82.7	75.4	89.8	81.7	59.9	98.4	3.5	1.8	0.66

[a]Standardized digestibility data were obtained by Parsons (1986) in cecectomized cockerels (n = 5 per sample) using a protein-free diet for determining basal endogenous losses.
[b]n = 3 per sample.

phenomenon, or a result of processing and guanidination of the animal protein.

Guanidination of animal proteins was conducted under high alkaline pH over a period of 4 d. Such conditions are known to result in carbonyl amine reactions (Swaisgood and Catignani, 1991), racemization (Swaisgood and Catignani, 1991; Piva et al., 2001), and β-elimination of AA residues in animal protein (Piva et al., 2001). These alterations in peptide structure may have hindered the absorption of the digested peptides. Thus, it is believed that the presence of these small, unabsorbed peptides may be an artifact of the animal protein preparation used. Unless future research shows that a quantitatively important fraction of the small peptides are not absorbed in commercial feed ingredients, it follows from these results that for in vitro assays, all soluble peptides should be considered digestible, independent of their size.

Comparison of the in vivo digestion of predigested and native guanidinated animal meal showed the same distribution of unabsorbed peptides, suggesting a similar extent of digestion. However, due to a problem with chromium in the predigested samples, actual digestibility coefficients could not be compared. One alternative method for determining whether in vivo digestion is exhaustive is to compare digestibility data from exhaustively digested in vitro samples with in vivo data. Using this assumption and the assumption that all soluble proteins should be considered digested, the in vitro AA digestibility coefficients for 25 animal meal samples were comparable to in vivo ones for lysine ($P = 0.71$), methionine ($P = 0.18$), threonine ($P = 0.26$), and valine ($P = 0.66$) (Table 5), suggesting that exhaustive digestion is appropriate. However, in vitro digestibility was lower for histidine ($P = 0.05$), isoleucine ($P < 0.01$), leucine ($P < 0.01$), and phenylalanine ($P = 0.05$). Also, no correlation between in vivo and in vitro digestibility for lysine was observed (Figure 3). This suggests that in vitro digestion was more variable (less precise) and, for some AA, less complete (less accurate) than in vivo digestion. Possible candidates carrying out the further digestion in vitro are pepsin, pancreatic proteases, and amino-peptidases. It is not known whether the amino-peptidases can digest insoluble peptides/protein, but if the in vitro digestion is indeed exhaustive, as demonstrated with the o-phthaldialdehyde assay (Qiao, 2001), then a possible implication of this work is that amino-peptidases can digest the insoluble peptides.

Another more likely explanation for the discrepancy between in vivo and in vitro digestibility is solubility problems in the in vitro assay. In the current in vitro assay, the solubilization medium was the water-based incubation medium with a pH of approximately 8, in which proteins were present at high concentration (5 mg/mL). In contrast, in vivo peptides are continuously removed and the concentrations may not reach the levels used in vitro, averting solubility problems. To test this hypothesis, differences between in vitro and in vivo

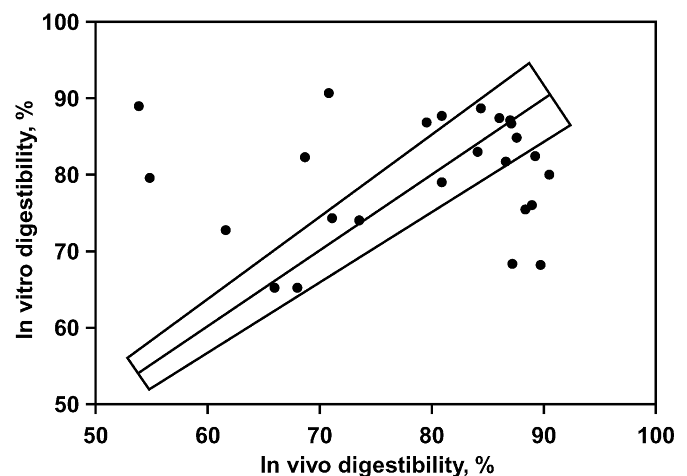

Figure 3. Lysine digestibility coefficients in vivo (n = 5 per sample) vs. in vitro (n = 3 per sample). The trapezoid indicates the target line (center line) ± 5%. Although the in vitro method was accurate, it was not precise because the digestibility of only half the samples was predicted with an error of less than 5%.

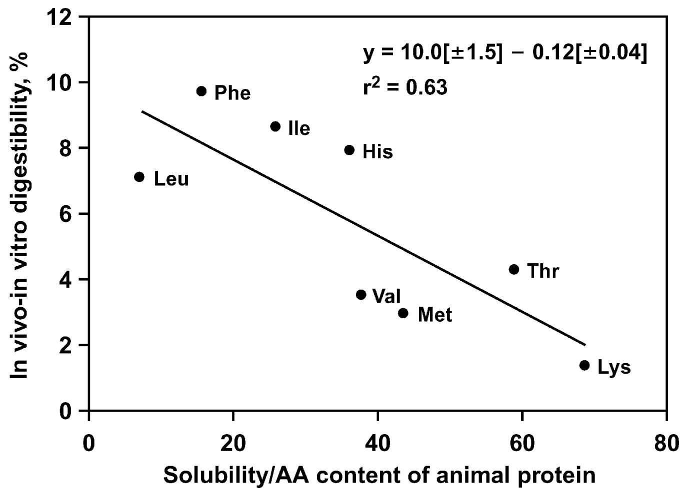

Figure 4. Discrepancy between in vivo and in vitro digestibility (n = 25) as a function of the concentration (%)-corrected solubility (g/L) of each amino acid.

digestibility across the 25 reference samples were regressed against AA solubility in water (Merck Index, 1989), corrected for the relative concentration of the AA in animal protein (average for the 25 reference samples). This regression showed that the higher the solubility, the lower the discrepancy between in vivo and in vitro digestibility (Figure 4). For future experiments, using an incubation medium that averts solubility problems may be key to resolving discrepancies between in vivo and in vitro digestibility assays. An example of such a medium is buffer supplemented with 2 *M* urea, as was used by Swaisgood and Horton (1989).

In addition to solubility problems, sample size and sample homogeneity are factors that may affect the outcome of the in vitro assay. The in vitro assay was developed using 0.5 g of animal protein as the starting material. Because this material was not finely ground before digestion (to avoid effects of grinding on digestibility), the size of this sample was likely insufficient to guarantee that the sample assayed was an aliquot of the entire sample. Data presented here suggest that the measurement error for the in vitro assay ranges from 4 to 8%, depending on the AA (Table 4). This error is large relative to the variation in digestibility coefficient observed (7 to 10%) in vivo, indicating that the repeatability of the in vitro assay and the corresponding AA assay needs to be improved. Selection of larger samples and/or grinding the animal proteins prior to sampling is recommended for future experiments, as well is running the in vitro assay in at least quadruplicate, as is done for the in vivo digestibility assays.

Although the current in vitro assay was not sufficiently precise to match in vivo data on an individual sample basis, the results are nevertheless much closer than those obtained with other published assays (Brule and Savoie, 1988; Savoie et al., 1989; Valette et al., 1993). This progress is due to more complete digestion in our assay and a more appropriate separation method. The referenced assays had short periods of incubation (at most 6 h for pepsin incubation and 24 h for pancreatic protease incubation) compared with our assay, but in these assays, peptides greater than 1 kDa were assumed to be indigestible, resulting in an underestimation.

Size separation of the ileal peptides has been performed previously (Asche et al., 1989a,b). Because the feed protein used by Asche et al. (1989a,b) was not labeled, and because the peptides they examined contained unabsorbed feed peptides and endogenous peptide losses, the size distribution did not reflect the property of the unabsorbed peptides of the feed. However, comparison of Asche's data to our results may yield information on how large the endogenous peptides are. Asche et al. (1989a,b) found that over one-third of the soluble ileal peptides were greater than 15 kDa, and the largest peptides were 500 kDa. In our study, soluble ileal peptides greater than 15 kDa were observed, but these peptides did not contain any homoarginine. This comparison suggests that the large soluble peptides found in the ileum are of endogenous origin. These endogenous ileal peptides probably were so large in molecular weight because they came from a region (e.g., lower portion of the small intestine) with insufficient proteolytic activity. The comparison thus suggests that predicting endogenous AA losses through acid precipitation of peptides larger than 10 kDa (Donkoh and Moughan, 1999) may result in an underestimation of the endogenous losses. A more appropriate threshold may be to measure all soluble proteins.

This study also yields insight into the validity of using guanidinated samples for studying digestibility. It had been expected that the most easily digestible lysine would be guanidinated first. The in vitro assay did not yield any evidence for this because the in vitro digestibility of lysine and homoarginine were very similar (Table 4), especially when correcting for enzyme contamination. One problem that was observed in this study was the large fraction of small homoarginine-containing peptides at the end of the ileum that was not absorbed. This study cannot discern if these peptides are a result of the guanidination process, but given that other research has not shown large fractions of small peptides in ileal content, it is logical to assume that the guanidination contributed to the occurrence of such peptides. Guanidination also seemed to affect the palatability of the test diets, as all animals ate their diets during the adaptation period, but 3 out of 18 meals containing guanidinated animal meals were refused. In this study, guanidination was used as a tool to generate feed ingredients suitable for comparing in vitro and in vivo digestibility, not for extrapolating data to unguanidinated animal meals. Thus, the validity of the guanidination procedure is not of major concern; however, the data do suggest that further validations are re-

quired of guanidination as a technique for assessing real digestibility of commercial feed ingredients.

Implications

No large soluble peptides (>1 kDa) derived from the feedstuffs were observed in ileal juices, suggesting that any protein solubilized in vitro is degraded into small peptides of less than 1 kDa in vivo. Not all the small homoarginine-containing peptides, however, were absorbed, likely because these peptides were damaged during guanidination. For in vitro assays, proteins should be exhaustively digested, and soluble peptides should be used as a measure of digestibility. This leads to a better in vitro digestibility assay than currently existing ones. The in vitro digestibility coefficients obtained with the assay described, however, did not match in vivo digestibility coefficients precisely for all individual samples and all amino acids, likely as a result of peptide solubility and sample homogeneity problems and insufficient repeats of the in vitro assays.

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