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A New Regression Equation of pH Drop Procedure for Measuring Protein Digestibility

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Abstract

A regression equation was proposed for predicting protein digestibility using pH drop and free amino acid content. Results were compared with those determined by the pH drop method of Satterlee et al. and with apparent *in vivo* digestibility in rats. Measured free amino acid content prior to four enzyme digestion had an influence on calculating digestibility. Results from new equation correlated more highly (r=0.8434, difference average=2.304) with *in vivo* digestibility than the results of pH drop method (r=0.7603, difference average=10.099).

Key words: regression equation, *in vitro* protein digestibility, pH drop procedure

INTRODUCTION

Yet it is known that the digestibility of a protein will have a significant impact on the nutritional quality of protein, the rat bioassay is the only official method for measuring food protein digestibility. Since evaluation of the protein digestibility of food products by *in vivo* method is very slow and costly, therefore, a sensitive, quick and reliable *in vitro* method is needed to replace them. Many methods have been developed and tested (1-5) for measuring *in vitro* protein digestibility to satisfy those purpose but some improvements in design and consideration in factors are still needed before those techniques can be used routinely. Satterlee and co-workers (6,7) have proposed a procedure in which protein digestibility is calculated from the drop in pH obtained after 20 minutes *in vitro* digestion using four proteolytic enzymes. They observed the pH drop was highly correlated with apparent *in vivo* digestibility in rats. However, several workers seriously questioned whether there was a direct relationship between the observed pH drop and protein hydrolysis (8-11). The other investigators also reported a greater discrepancies and poor correlation coefficients between the *in vitro* digestibilities from pH drop procedure and *in vivo* results in rats when the animal based foods, fermented proteins and viscous protein samples were used (12-17).

The purpose of our study was to design a new regression equation of pH drop procedure for determining *in vitro* protein digestibility which could give much closer estimate of the *in vivo* digestibility than the pH drop procedure of Satterlee et al. (6). Since the pH drop obtained on hydrolysis of a protein is dependent on substrate level and buffering capacity of hydrolyzates, we checked initial pH of sample solutions and determined free amino acid contents prior to digestion. A new regression equation was designed by multiple regression method in considerations of final pH after four enzyme digestion, initial pH prior to digestion, pH difference between initial and terminal pH, and free amino acid content of the identical samples used in rat bioassay.

MATERIALS AND METHODS

Materials

Protein sources used in this study were fish meats of live loach (*Misgurnus anguillicadatus*), crucian carp (*Carassius carassius*), bastard halibut (*Paralichthys olivaceus*), jacopever (*Sebastes schlegeli*) and their meat extracts. All of the samples were prepared as eviscerated and scaled, and then freeze dried solids with a particle size sufficiently small to pass an 80-mesh screen. Trypsin (Sigma, 14,600 BAEE unit/mg solid), α-chymotrypsin (Sigma, 41 units/mg solid), peptidase (Sigma, 500 units/

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mg solid) and bacterial protease (*Streptomyces griceus*, Sigma, 58 units/mg solid) for *in vitro* protein digestibility assay were purchased from Sigma. ANRC casein, vitamin mixture, and mineral mixture were purchased from ICN Biomedicals Inc. Corn starch from Shindongbang Inc. and corn oil from Cheil Jedang were also used in rat diets.

In vivo apparent digestibility determination

The 21~22 day old male weanling albino rats (Sprague-Dawley) were placed into individual cages. The rats were housed in a room maintained at 22~24°C, 50~60% RH with alternating 12 hour periods of light and dark. Rats were placed on an adaptation diet for 4 days, weighed at the end of the adaptation period, and then randomly distributed to experimental groups (10 rats per group). Each groups was fed an experimental diet containing 10% protein for 28 days. To reduce the quality deterioration of diets from lipid oxidation, diets were stored in -20°C refriegerator as the airtight individual small packs for daily consumption throughout experiments. Food and water were supplied *ad libitum*. These data were collected during routine protein efficiency ratio (PER) tests (18). Food consumption was measured throught the study, and feces were collected for eight days (days 18~26). A control diet of ANRC casein was included in each study. The micro-kjeldahl procedure (19) was used for nitrogen determinations.

$$\% \text{ Apparent digestibility} = \frac{\text{N intakes (g)} - \text{N in feces (g)}}{\text{N intakes (g)}} \times 100$$

Determination of *in vitro* digestibility

The *in vitro* digestibility was determined, in duplicate, by the improved pH-drop procedure of Satterlee et al. (6), and checked the initial pH of sample dispersed in glass distilled water before four enzyme digestion to facilitate later discussion.

Quantification of free amino acid content

Free amino acid content was measured by the spectrophotometric procedure (20,21) using *o*-phtaldialdehyde (OPDA) reagent. Results were calculated as DL-leucine and DL-lysine equivalents

Regression analysis

In vitro parameters were defined as (X_1) pH difference between initial pH before digestion and pH 8, (X_2) final pH after the 20 min digestion, (X_3) free amino acid content by OPDA method expressed as DL-leucine equivalent, and (X_4) free amino acid content by OPDA method expressed as DL-lysine. We executed all-possible-regressions selection procedure as variable selection method. When this selection procedure was used with our data, 15 kinds of different regression models are to be considered.

Two criteria for comparing the various regression models such as Rap^2 (adjusted coefficient of multiple determination) and Cp were used in those procedure. We sought to find the subset of X variables that could maximize Rap^2 and minimize Cp value close to P. The programme of SAS (22) was run to determine the multiple regression equation.

RESULTS AND DISCUSSION

In vivo and *in vitro* protein digestibilities

Table 1 gives data for the pH of each sample following the 20 min digestion of pH-drop assay. Since Satterlee et al. (6) had shown earlier that this 20 min pH and its drop from 8.0 reflected the degree of protein digestibility data, those pH data was compared against the *in vivo* digestibility data for each seafood protein sources. But it could be noted that the pH drop assay still tends to under-predict the percent digestibility of seafood based proteins. It was found that *in vitro* digestibility of pH-drop assay showed a lower correlation (r=0.760) with *in vivo* apparent digestibility. Those seafood proteins which have been improperly heat treated resulted the greater discrepancies between in the *in vitro* and *in vivo* digestibility (12). The relatively poor correlation could be attributed to the low level of proteins for substrate of enzymatic digestion. Therefore, the more accurate or improved assessment of protein digestibility is needed to critically evaluate seafood proteins.

In vitro parameters of seafood proteins

Because of the pH drop obtained on hydrolysis of a protein is dependent on factors other than extent of hydrolysis due to free carboxyl groups and cannot be used as a measure of the extent of protein hydrolysis, the other factors that could lead to discrepancies in results obtained for comparison of protein digestibility by the pH drop method should be considered. Especially

Table 1. Comparison of *in vitro*[1] digestibilities with *in vivo* digestibilities of fish protein sources

Diet	pH at 20 min.	*In vitro* dig.(%)	*In vivo* dig.(%)
ANRC casein	6.36	90.00	89.88
Raw bastard halibut	6.84	80.53	92.59
Raw jacopever	6.73	83.01	91.12
Raw crucian carp	6.80	81.43	93.90
Raw loach	6.68	84.14	89.86
HEH	7.43	67.22	85.25
JEH	7.41	67.67	75.26
CEH	7.49	65.87	76.31
LEH	7.37	68.57	79.33
HGEM	7.23	71.73	85.31
JGEM	7.10	74.66	86.23
CGEM	7.19	72.63	86.48
LGEM	7.21	72.18	87.01
HEA	7.01	76.69	86.13
JEA	6.97	77.60	85.41
CEA	6.85	80.30	84.91
LEA	6.95	78.05	90.50
HGEA	7.01	76.69	87.69
JGEA	6.85	80.30	85.26
CGEA	6.86	80.08	86.90
LGEA	6.99	77.15	83.04

[1]Using pH drop assay of Satterlee et al. (6,7)
HEH: halibut extracts processed at 140°C for 9.85 hours
JEH: jacopever extracts processed at 140°C for 9.38 hours
CEH: crucian carp extracts processed at 136.7°C for 7.25 hours
LEH: loach extracts processed at 140°C for 10.08 hours
HGEM: (halibut+ginger) extracts processed at 110°C for 5 hours
JGEM: (jacopever+ginger) extracts processed at 110°C for 5 hours
CGEM: (crucian carp+ginger) extracts processed at 110°C for 5 hours
LGEM: (loach+ginger) extracts processed at 110°C for 5 hours
HEA: halibut extracts processed at 100°C for 6 hours
JEA: jacopever extracts processed at 100°C for 6 hours
CEA: crucian carp extracts processed at 100°C for 6 hours
LEA: loach extracts processed at 100°C for 6 hours
HGEA: (halibut+ginger) extracts processed at 100°C for 6 hours
JGEA: (jacopever+ginger) extracts processed at 100°C for 6 hours
CGEA: (crucian carp+ginger) extracts processed at 100°C for 6 hours
LGEA: (loach+ginger) extracts processed at 100°C for 6 hours

different buffering capacities of proteins and substrate concentration would be expected to involve the those discrepancies (8,9). Differences in protein buffering capacities could be caused by the presence of salts, phenolic acids, etc., and/or exposure of previously buried unprotonated basic groups as proteins are hydrolyzed (9). But this is less of a problem with pH drop method that was unaffected by the buffers normally present in food (5). Thus, rupture of peptide bonds would be considered as the predominant source of protons released and of the associated pH change during enzymatic hydrolysis of proteins. Therefore, we observed the initial pH of seafood samples dispersed in glass distilled water and measured the free amino acid content of those original protein samples to check the actual substrate levels for enzymatic digestion of pH drop method (Table 2). Considerable variation between seafood proteins was observed in the all *in vitro* parameters and *in vivo* digestibilities. In general, the higher *in vivo* digestibilities of seafood samples resulted the lesser gap between initial pH and pH 8, and then yielded the lower terminal pH after enzyme digestion. Those samples also had the lower level of free amino acid contents when compared with those of low *in vivo* digestibility samples. This suggests that there was not a severe thermolysis arisen in those kinds of seafood samples and then resulted the greater pH drop. In support of these we observed that a direct relationship between those *in vitro* parameters and *in vivo* digestibility may be apparent.

Regression analysis

The possible *in vitro* parameters that could affect digestibility, as discussed in previous paragraph with Table 2, were used in regression analysis. In order to determine the best effective variables using variable selection method, 15 kinds of regression models were considered as shown in Table 3. 0.52867 of Rap^2 (adjusted coefficient of multiple determination) could result Cp criteria (1.9244) close to P (2) using variables X_2 (terminal pH at 20 min. digestion of pH-drop method) and X_3 (free amino acid content expressed as a D-leu. equivalent). The largest value of Rap^2 from X_2 and X_3 indicated that those variables were the most important variables influencing the prediction of *in vitro* digestibility. The best explanatory model equation for *in vitro* digestibility is shown in Table 4 as follows; Predicted *in vitro* digestibility (%)=151.944015-8.78545 · X_2-1.138901 · X_3. After the analysis of variance, F value was 12.217 (Prob>F: 0.0004) which considered adequate with satisfactory *in vitro* protein digestibility close to *in vivo* digestibility.

Table 2. *In vivo* digestibility and *in vitro* parameter of protein sources

Diet	*In vivo* dig. (%)	8-Initial pH (X_1)	Terminal pH (X_2)	Free amino acid (% dry base)	
				DL-Leu (X_3)	DL-Lys (X_4)
ANRC casein	89.88	0.09	6.36	0.15	0.06
Raw bastard halibut	92.59	1.20	6.84	1.63	1.40
Raw jacopever	91.12	1.15	6.73	2.25	1.94
Raw crucian carp	93.90	0.69	6.80	2.06	1.78
Raw loach	89.86	1.00	6.68	3.18	2.73
HEH	85.25	1.67	7.43	4.83	4.15
JEH	75.26	1.65	7.41	4.78	4.10
CEH	76.31	1.87	7.49	4.74	4.07
LEH	79.33	1.84	7.37	4.76	4.09
HGEM	85.31	1.55	7.23	3.81	3.28
JGEM	86.23	1.89	7.10	3.88	3.33
CGEM	86.48	0.88	7.19	3.63	3.04
LGEM	87.01	1.76	7.21	3.26	3.04
HEA	86.13	1.80	7.01	4.10	3.51
JEA	85.41	1.88	6.97	3.84	3.30
CEA	84.91	1.65	6.85	4.63	3.98
LEA	90.50	1.76	6.95	4.44	3.81
HGEA	87.69	1.99	7.01	4.43	3.80
JGEA	85.26	1.82	6.85	3.88	3.34
CGEA	86.90	1.57	6.86	4.60	3.95
LGEA	83.04	1.76	6.99	4.47	3.84

Samples are as same as in Table 1

In vivo digestibility and predicted *in vitro* digestibility of seafood proteins

Predicted *in vitro* protein digestibilities estimated by new regression equation and *in vitro* digestibilities of Satterlee et al. (6) were listed in Table 5 in comparing against the *in vivo* digestibilities. Even though the new equation for *in vitro* digestibility was built up of terminal pH at 20 min. digestion and free amino acid content, those equation still gave the lower estimates of digestibility in most of seafood protein sources when compared with *in vivo* digestibility of same sample like as the equation from pH-drop method of Satterlee et al. (6). But the new *in vitro* determinations of digestibility had a high degree of correlation with *in vivo* findings (r=0.8434), whereas those of the pH-drop method had a relatively

Table 3. Rap^2 and Cp values for all-possible-regressions selection procedure

X	P	Rap^2	Cp
X_1	1	0.239208	11.8361
X_2	1	0.508040	1.6466
X_3	1	0.412334	5.2741
X_4	1	0.406314	5.5023
$X_1 \cdot X_2$	2	0.491014	3.2766
$X_1 \cdot X_3$	2	0.382268	7.1814
$X_1 \cdot X_4$	2	0.377418	7.3556
$X_2 \cdot X_3$	2	0.528671	1.9244
$X_2 \cdot X_4$	2	0.523186	2.1214
$X_3 \cdot X_4$	2	0.383768	7.1276
$X_1 \cdot X_2 \cdot X_3$	3	0.507250	3.7106
$X_1 \cdot X_2 \cdot X_4$	3	0.500949	3.9243
$X_1 \cdot X_3 \cdot X_4$	3	0.348023	9.1104
$X_2 \cdot X_3 \cdot X_4$	3	0.528192	3.0004
$X_1 \cdot X_2 \cdot X_3 \cdot X_4$	4	0.498717	5.0000

X: variable in model
P: number of selected variables
Rap^2: adjusted coefficient of multiple determination
Cp: criteria
X_1~ X_4 means the *in vitro* parameter as shown in Table 2

Table 4. Regression analysis for multiple regression equation using *in vitro* parameters

Adjusted coefficient of multiple determination (Rap^2): 0.528671
Cp criteria: 1.9244
Number of selected variable (P): 2
Optimum variable in model: X_2, X_3
Regression equation: $Y=151.944015-8.785450 \cdot X_2-1.138901 \cdot X_3$
where Y is predicted *in vitro* dig. (%)
X_2 is final pH at 20 min. digestion
X_3 is free amino acid content expressed as D-leu. equivalent
Analysis of variance
F value: 12.217
Prob>F: 0.0004

Table 5. *In vitro* digestibilities of protein sources compared with *in vivo* digestibility

Diet	*In vitro* dig. (%)		*In vivo* dig. (%)
	Satterlee et al.	New model	
ANRC casein	90.00	95.8977	89.88
Raw bastard halibut	80.53	89.9951	92.59
Raw jacopever	83.01	90.2554	91.12
Raw crucian carp	81.43	89.8568	93.90
Raw loach	84.14	89.6355	89.86
HEH	67.22	81.1672	85.25
JEH	67.67	81.3999	75.26
CEH	65.87	80.7426	76.31
LEH	68.57	81.7741	79.33
HGEM	71.73	84.0860	85.31
JGEM	74.66	85.1484	86.23
CGEM	72.63	84.6424	86.48
LGEM	72.18	84.8881	87.01
HEA	76.69	85.6885	86.13
JEA	77.60	86.3360	85.41
CEA	80.30	86.4906	84.91
LEA	78.05	85.8284	90.50
HGEA	76.69	85.3127	87.69
JGEA	80.30	87.3447	85.26
CGEA	80.08	86.4369	86.90
LGEA	77.15	85.4428	83.04
Ave. dif.	10.099	2.304	

Samples are same as in Table 2
Satterlee et al.: calculated *in vitro* digestibility (%) using the equation of Satterlee et al. (6)
New model: determined *in vitro* digestibility (%) using the new multiple regression equation of this study
Ave. dif.: average of difference between *in vitro* and *in vivo* digestibilities

lower correlation coefficient (r=0.7604). The accuracy of the digestibility data, as predicted from new equation, was 4.38 times better than that obtained with the pH-drop method of Satterlee et al. (6) when compared on difference average (new equation 2.304<pH-drop method 10.099). On the basis of our results observed previously, modified pH-drop method of our study may have practical significance and advantages for measuring the digestibility of proteins which had been already digested and/or showed high levels of free amino acid contents before four enzyme digestion.

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