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Registry No. Formononetin, 485-72-3; daidzein, 486-66-8; equol, 531-95-3; 1-naphthol, 90-15-3.

Protein Digestibility of Alkali- and Fructose-Treated Protein by Rat True Digestibility Assay and by the Immobilized Digestive Enzyme Assay System†

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The effect of processing on the digestibility of various food proteins was examined by using the immobilized digestive enzyme assay (IDEA) system. The values obtained were compared to true digestibilities determined by rat bioassay. Sodium caseinate, egg white, soy protein, and whey were treated with either 0.2 N NaOH at 40 °C for 6 h or 0.5 M fructose (pH 7.0) at 90 °C for 4 h. Untreated proteins were also analyzed. Treatment of all samples ($n = 12$) with 4 M urea assured solubility. Regression analysis of data for all samples resulted in a correlation coefficient (r) of 0.83 ($p < 0.001$). The IDEA systems appears to be an accurate and reliable estimate of in vivo digestibilities. Furthermore, it offers a more rapid and less expensive alternative to animal bioassays.

The immobilized digestive enzyme assay (IDEA) system has been used as an in vitro measure of protein digestibility (Porter et al., 1984) and in detection of decreases in protein digestibility due to protein modifications (Chung et al., 1986). Porter et al. (1984) reported that the IDEA system gave digestibilities in agreement with FAO and literature values for a number of plant and animal proteins. In addition, it has been shown that loss of digestibility was correlated to degree of racemization, to lysinoalanine formation, and to loss of available lysine, indicating that the system is sensitive to protein modification resulting from alkali treatment or Malliard reactions (Chung et al., 1986).

In a cooperative study on assessment of protein nutritive value (Bodwell et al., 1989), 17 commonly consumed foods were analyzed for various parameters of protein quality with several in vivo and in vitro measurements. As part of that study, Thresher et al. (1989) using the IDEA system compared the protein digestibility of the foods to two independent in vivo estimates of digestibility (Eggum et al., 1989; Sarwar et al., 1989). Although good agreement was seen between digestibilities for some foods, true comparisons were hampered by the lack of protein solubility.

These observations suggested that the IDEA system might represent a rapid, facile and inexpensive predictor of protein digestibility compared to in vivo methods where complete protein solubility could be achieved. This report gives the results of those comparisons.

EXPERIMENTAL SECTION

Materials. Porcine pepsin, trypsin, chymotrypsin, and intestinal peptidases were obtained from Sigma Chemical Co. The intestinal peptidase was further purified by the method of Porter et al. (1984). Sodium caseinate, egg white, and soybean protein were obtained from U.S. Biochemicals. Whey protein (Vari-Dairy Plus) was obtained from Nutrisearch. Succinic anhydride, trimethylamine, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC), fructose, benzoyl-L-tyrosine ethyl ester, (BTEE), (*p*-toluenesulfonyl)-L-arginine methyl ester (TAME), L-leucylglycine, urea, and controlled-pore glass beads

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(198-nm pore diameter, 120–180 mesh) were obtained from Sigma. β-Mercaptoethanol was obtained from Fisher Scientific, glycylglycylphenylalanylphenylalanine ethyl ester from Serva, (3-aminopropyl)triethoxysilane from Silar, o-phthalaldehyde (OPA) from Pierce, and sodium dodecyl sulfate from Bio-Rad. All other chemicals were reagent grade.

Enzyme Reactors and Determination of Digestibility. Aminopropyl glass beads were prepared as described by Janolino and Swaisgood (1982) and succinylated with succinic anhydride (0.1 mg/mL) dissolved in chloroform/triethylamine (23/1, v/v).

Pepsin was immobilized on the succinamidopropyl glass beads by a sequential activation/immobilization procedure (Janolino and Swaisgood, 1982). Surface carboxyl groups were activated with 10 mM EDC at pH 4.75 for 20 min at room temperature and rapidly washed (<2 min) with cold 0.5 M NaCl, and a pepsin solution (5 mg/mL) adjusted to pH 4.65 was recirculated through a column of the beads at 4 °C overnight. The immobilized pepsin was washed successively with HCl (pH 2), 4 M urea, and HCl (pH 2). Trypsin, α-chymotrypsin, and intestinal peptidases were immobilized as described by Porter et al. (1984).

Activities of the immobilized enzymes were assayed with a microrecirculation reactor (Taylor and Swaisgood, 1980) as described by Chung et al. (1986). In vitro digestibilities were calculated as the fraction of peptide bonds hydrolyzed following treatment with immobilized pepsin and immobilized trypsin, chymotrypsin, and intestinal peptidase bioreactors according to the procedures described by Porter et al. (1984) and Chung et al. (1986). Released α-amino groups were quantitated by reaction with o-phthalaldehyde. Between assays, the bioreactors were regenerated by washing with 4 M urea (Chung et al., 1986).

Protein Treatments. Preparation of the protein samples is summarized in Figure 1. For the alkali treatment, sodium caseinate, egg white, soy, and whey proteins were dissolved in 0.2 M NaOH at a concentration of 50 mg/mL. A 500-mL portion of each solution was placed in flasks and heated in a water bath at 40 °C for 6 h. For fructose treatment, the four proteins were dissolved (50 mg/mL) in 0.01 M sodium phosphate (pH 7.0) containing 0.5 M fructose, placed in flasks, sealed, and heated in a water bath at 90 °C for 4 h. After both sets of samples had cooled, the treated samples and solutions of untreated proteins (50 mg/mL) were made 4 M in urea, adjusted to pH 3.5 with dilute HCl, and filtered through Whatman No. 2 paper under vacuum. The filtrates were concentrated to 50 mL with an Amicon TCF 10 concentrator using a YM 10 membrane. The concentrated samples were dialyzed against 0.01 N HCl for 72 h at 4 °C and lyophilized.

Rat Bioassays. The procedure described by Bodwell et al. (1980) was used. Male weanling Sprague–Dawley rats were maintained on a 20% casein diet (AIN-76A) for 7 days. The rats were housed individually in stainless steel screen-bottom cages in an animal room maintained at 70 °F with a daily light cycle of 12 h light and 12 h dark. Feed and water were provided ad libitum. Eleven rats were then placed in metabolic cages and fed the respective test diet for 12 days. The last 5 days of this period, feed consumption was measured and feces were collected. Five rats were fed diets containing the test protein at a 10% level (N × 6.25 or 6.38). For determination of obligatory fecal nitrogen losses, six rats were fed a 3% egg protein diet (Bricker and Mitchell, 1947). This diet was kept isocaloric to the test protein diets by substituting an additional 7% starch. Feces were collected into a dilute sulfuric acid solution daily and stored at −20 °C. At the end of the 5-day collection period, fecal samples were composited, dried to a constant weight at 50 °C for 48 h, and ground. Diet and fecal samples were analyzed for nitrogen by Kjeldahl. True digestibility was calculated as follows: $TD = I - (F - F_0)/I$, where I is nitrogen intake, F is fecal nitrogen loss for the test protein group, and F_0 the fecal nitrogen loss for the obligatory nitrogen loss group.

Statistical Analyses. Regression and statistical analyses were performed according to SAS (1985) procedures.

RESULTS AND DISCUSSION

The activities of the immobilized enzymes are shown in Table I. While activities may vary slightly between immobilization procedures, similar values are generally obtained (Porter et al., 1984; Chung et al., 1986). Slight variations do not affect the results of the digestibility assay since it is a measure of extent of hydrolysis (reactions reach completion) as opposed to an initial rate of reaction assay.

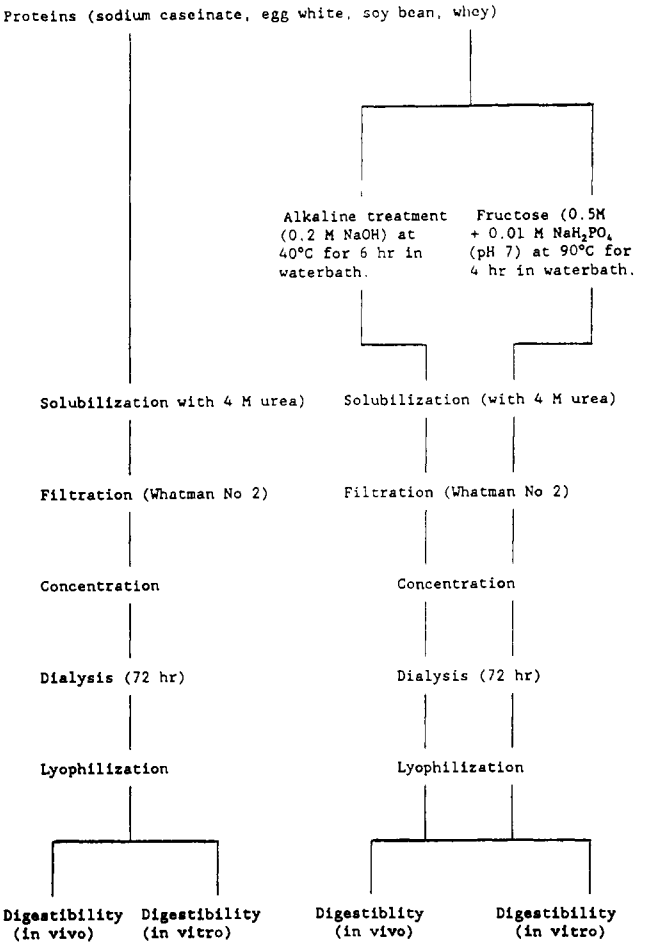

Figure 1. Flow chart for the preparation of the protein samples.

Table I. Activity of Immobilized Enzymes

enzyme	substrate	act., μmol·min^{-1}/g beads
pepsin	GlyGlyPhePheOEt	0.74
trypsin	TAME	46.80
chymotrypsin	BTEE	19.13
peptidase	LeuGly	0.41

The in vivo and in vitro assay data for the protein digestibility of the twelve test proteins are shown in Table II. Both alkali and fructose treatments reduced the digestibility of the four proteins tested. A comparison of the two methods by regression analysis (Figures 2) indicates that a linear relationship exists with a correlation coefficient of 0.83 ($p < 0.001$). The regression equation is $y = 81.143 + 0.2419x$. Comparison of the IDEA system digestibility values with in vivo digestibility of commonly consumed foods (Thresher et al., 1989) showed similar regression equations, $y = 79.81 + 0.3190x$, $r = 0.76$ (Eggum et al., 1989), and $y = 82.22 + 0.2830x$, $r = 0.80$ (Sarwar et al., 1989). Table II also shows the predicted in vivo digestibilities calculated from the regression equation obtained from the present study. These results suggest that the IDEA system represents an accurate and reliable predictor of in vivo digestibility.

The IDEA system gave the least reliable value for unprocessed egg white. The presence of appreciable

Table II. Digestibility of Unprocessed and Processed Proteins by in Vivo and in Vitro Systems

sample		digestibility IDEA,[a] %	actual digestibility in vivo,[b] %	predicted digestibility in vivo,[c] %
casein	U[d]	55.3 ± 0.13	94.7 ± 0.32	94.5 ± 1.00
	A[e]	43.6 ± 0.09	91.7 ± 0.80	91.7 ± 0.58
	F[f]	36.1 ± 0.12	90.2 ± 0.56	89.9 ± 0.55
egg white	U	40.3 ± 0.10	95.1 ± 0.67	90.9 ± 0.54
	A	28.1 ± 0.08	88.9 ± 0.63	87.9 ± 0.77
	F	31.9 ± 0.17	89.9 ± 0.55	88.9 ± 0.64
soybean	U	50.2 ± 0.22	91.7 ± 0.31	93.3 ± 0.79
	A	27.2 ± 0.14	85.1 ± 0.38	87.7 ± 0.81
	F	36.1 ± 0.15	88.4 ± 0.21	89.9 ± 0.55
whey	U	57.0 ± 0.21	94.1 ± 0.48	94.9 ± 1.08
	A	26.7 ± 0.16	86.7 ± 0.22	87.6 ± 0.83
	F	34.5 ± 0.09	90.2 ± 0.23	89.5 ± 0.58

[a] Each value is the average of four replicates. Means ± SD. [b] Values are the average from five rats. Means ± SD. [c] Predicted values were calculated from the regression equation shown in Figure 2. Means ± SD. [d] Untreated protein samples. [e] Alkali-treated proteins were incubated for 6 h in 0.2 M NaOH at 40 °C. [f] Fructose-treated proteins were incubated for 4 h in 0.5 M fructose at pH 7.0 and 90 °C.

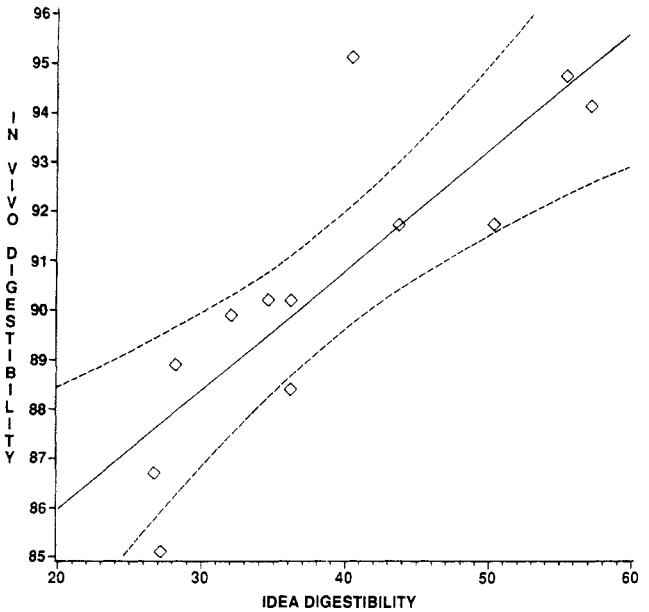

Figure 2. Relationship between digestibility as determined by rat bioassay and the IDEA system. The solid line represents the linear regression given by $y = 81.143 + 0.2419x$, $r = 0.83$. The dashed line represents the 95% confidence interval for the true line.

amounts of glycoproteins could sterically affect proteolysis in a manner similar to decreased hydrolysis observed for sugar–protein adducts formed during browning. The inclusion of glycolytic enzyme(s) in the second reactor might improve hydrolysis of such proteins. In addition, egg white contains approximately 4% lysozyme, a protein with considerable structural stability (stable in 4 M urea). Since both the alkali and fructose treatments of egg white produced accurate IDEA digestibilities compared to in vivo estimates, it is possible that both treatments produced sufficient destabilization to overcome residual hydrolytic resistance.

Alkali, fructose, and heat treatments produced differential solubilities among proteins, compared to controls. While these protein could have been used directly in the in vivo assay, the IDEA reactors, in a fixed-bed configuration cannot be used with insoluble (or particulate) substrates. Therefore, in order to make a direct comparison of digestibility with both systems, the treated proteins were resolubilized with 4 M urea (Figure 1). It should be noted that the use of urea (4 M) to ensure solubility of the proteins (with the possible exception of unprocessed egg white) did not affect the accuracy of the IDEA digestibilities and thus apparently does not represent a "nonphysiological" treatment of the proteins prior to assay. These data along with the use of a fluidized-bed reactor configuration to facilitate assay of particulate samples (Culver and Swaisgood, 1989) should make the use of the IDEA system on food products a reality.

The advantages of the IDEA system over other previously reported soluble enzyme in vitro digestibility assays have been pointed out (Chung et al., 1986). When compared to in vivo assays, the IDEA system represents a considerable savings in both time and cost. The rat bioassay used in this study took 19 days versus 2 days for the IDEA system. The costs of metabolic cages (necessary for quantitative collection of feces), animals, per diem charges for animals, and diet materials and formulation far outweigh that for materials (enzymes, chemicals, glass beads) and equipment (a peristaltic pump) needed for the IDEA system.

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