In Vitro Determination of Protein Digestibility in Wheat Millfeeds for Monogastric Animals[1]

R. M. SAUNDERS and G. O. KOHLER, Western Marketing and Nutrition Research Division, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, Calif. 94710

ABSTRACT

An *in vitro* method has been devised to measure protein digestibility in wheat millfeeds for monogastric animals. The *in vitro* system comprises treatment with a fungal protease followed by treatment with chick pancreas acetone powder. The method correlated with a feeding experiment and with other *in vitro* systems. Protein digestibility in wheat millfeeds is discussed.

Although the biological availability of protein in feeds must, in the final analysis, be established by exact feeding trials, *in vitro* methods of evaluating protein digestibility are important because of their rapidity and sensitivity. They are particularly useful where feeds are being processed in a large number of ways, and where animal feeding trials to assess the processing effects would necessarily be tedious, expensive, and incapable of detecting small differences. Several laboratory methods for chemical estimation of protein digestibility in feeds have been published (1,2,3). Most of these methods include a pepsin-amylase digestion of the feed under investigation, and this system has actually been extended to measure protein quality as well as protein digestibility (4,5). A recent review lists the possible errors involved with pepsin digestions and interpretations (6). We have devised another *in vitro* system for measuring protein digestibility which is described herein. The method appears particularly suitable for wheat millfeeds and products derived therefrom. Measurements of protein digestibility using this simple procedure agreed with measurements with other *in vitro* systems, and correlated with protein-digestibility measurements in rat feeding trials. The procedure requires a very small quantity of feed, and several dozen determinations can be performed simultaneously by the operator. This procedure and some of the results have been presented in part (7,8).

[1]Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

MATERIALS AND METHODS

Pronase B Grade (*Streptomyces griseus* protease) and crystalline trypsin (hog) were obtained from Calbiochem, Los Angeles. Chick pancreas acetone powder was prepared as previously described (9).

The wheat millfeeds, bran, shorts, red dog, germ, and patent flour were from the same blend of hard red spring wheat, and were donated by International Multifoods, through the courtesy of William Johnston. The materials were used as received and were not reground further.

The *in vitro* procedure for protein digestibility is as follows: 1 g. of material suspended in 15 ml. of water is heated on a boiling-water bath for 5 min. After cooling, 5 ml. of 0.02M sodium barbiturate buffer, pH 7, containing 0.15M sodium chloride (NaCl) and 5 mg. of pronase, is added. The mixture is gently shaken at 21°C. for 16 hr. Then solids are separated by centrifuging[2] and washed once with water. The solids are resuspended in 15 ml. of water and heated on a boiling-water bath for 5 min. After cooling, 5 ml. of barbiturate buffer and 5 mg. of chick pancreas acetone powder are added, and the mixture gently shaken at 21°C. for 16 hr. The solids are separated by centrifuging and washed with water (5 X 30 ml.), centrifuging and removing the supernatant after each washing. The solids are finally filtered through a 1.2 μ filter (Millipore), air-dried, weighed, and analyzed for nitrogen (N). The calculation is as follows: Protein digestibility (%) = (N in bran - N in indigested fragment)/(N in bran) X 100. Experiments are normally carried out in triplicate.

The rate of protein digestibility in bran by pronase was measured as follows: 1 g. of bran was mixed with 15 ml. of water and heated on a boiling-water bath for 5 min. After cooling, the mixture was diluted with 5 ml. of barbiturate-NaCl buffer and treated with 5 mg. of pronase. The mixture was dialyzed against 1 liter of water at 21°C. One-milliliter aliquots were removed at timed intervals from the dialysate and assayed for hydrolyzed protein by the Folin procedure (10). The absorbance values at 750 nm. for the 1-ml. aliquots were plotted directly in Fig. 2.

For the feeding trial, groups of five rats, Sprague-Dawley strain, initial age 22 days, were separately fed each ration. The diets, including the casein control diet (Table I), were formulated to contain 10% protein. These diets differed from the control by the various levels of carbohydrates, corn oil, and cellulose required to produce isocaloric and isonitrogenous diets when the wheat milling fraction supplied the total protein. A N-free diet was included in the test to measure metabolic fecal N; in this case the group of five rats were of initial age 36 days so that metabolic excretion of N was more closely related to the weight of the rats which had been on the tested diets for 1 to 2 weeks when the fecal collections were made. The rats were fed these rations *ad libitum* for 21 days. During the second week of the feeding trial, the feces were collected quantitatively from each group of rats and the moisture-free fecal weights and N content were determined. Protein digestibility (%) = (N in feed - [N in feces - metabolic N])/(N in feed) X 100.

Amino acid analyses of undigested protein after *in vitro* or *in vivo* (fecal) digestions were determined on acid hydrolysates (6N HCl for 24 hr.) by ion-exchange chromatography with a modified Phoenix amino acid analyzer (11).

[2] In this procedure, centrifuging means for a period of 2 to 3 min. at ~10,000 g.

TABLE I. COMPOSITION OF CASEIN CONTROL DIET

Ingredient	%	Ingredient	%
Casein	11.76	Cellulose	6.50
Corn oil	3.96	Vitamin mix[b]	2.00
Water	9.14	Corn starch	20.00
Salts[a]	3.77	Dextrose	42.87

[a]Salt mix: Salt mix U.S.P. XIV, Nutritional Biochemicals Corp., fortified with $ZnSO_4$ and $CoCl_2$.

[b]Vitamin mix: Vitamin diet fortification mixture, Nutritional Biochemicals Corp.

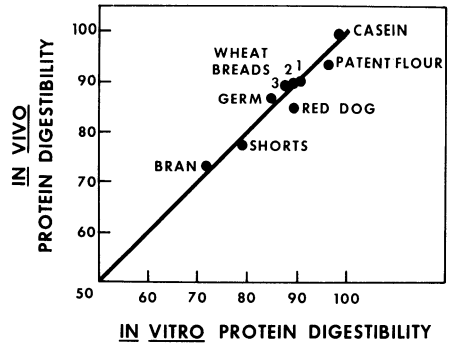
TABLE II. PROTEIN DIGESTIBILITY IN WHEAT MILLFEEDS MEASURED BY IN VITRO AND IN VIVO[a] PROCEDURES

Millfeed	Protein Digestibility % In Vitro	In Vivo
Casein control	98.5	99.3
Patent flour	96.7±1.3	93.2
Red dog	89.5±0.6	84.6
Germ	85.5±0.4	86.7
Shorts	79.1±0.3	77.4
Bran	71.5±0.6	73.0

[a]Corrected for metabolic-N excretion.

RESULTS AND DISCUSSION

Table II lists the measured protein digestibility by *in vitro* and *in vivo* procedures for wheat millfeeds and casein. The correlation is shown graphically in Fig. 1. Also shown in Fig. 1 are the protein digestibilities found by both systems for three whole-wheat breads, which indicates that this *in vitro* system can be extended to wheat-based products. The correlation coefficient is 0.962. The feeding trial here was very similar to that carried out by Summers et al. (12). The rate of protein hydrolysis in wheat bran with pronase is illustrated in Fig. 2. Under the conditions employed here the digestion effected by pronase has gone to completion in about 12 hr., i.e., extent of digestion and not merely rate of digestion is being measured in the standard *in vitro* procedure. A similar observation has been made in the case of the starch digestion. The initial heating in water with the recommended *in vitro* system has been shown to have no effect on the final protein-digestibility figures,



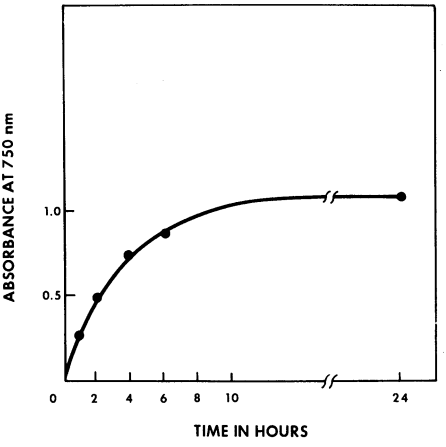


Fig. 1 (left). *In vitro* vis. *in vivo* measurement of protein digestibility in wheat millfeeds and three whole-wheat breads.

Fig. 2 (right). Rate of hydrolysis of wheat bran protein with pronase.

TABLE III. PROTEIN DIGESTIBILITY IN WHEAT MILLFEEDS MEASURED BY DIFFERENT IN VITRO PROCEDURES

Millfeed	Saunders & Kohler[a]	Booth & Moran (1)[b]	Chick et al. (2)[b]	Akeson & Stahmann (5)[b]
Patent flour	96.7	99.4	99.0	98.9
Red dog	89.5	95.2	94.4	93.9
Germ	85.5	91.5	92.8	88.3
Shorts	79.1	82.7	84.1	80.8
Bran	71.5	74.5	72.0	69.7

[a]Average values from triplicate determinations.

[b]Average values for duplicate determinations.

and may be unnecessary. However, an advantage of the heating step is that it should eliminate possible microbial contamination and its subsequent effects on the digestion. Although the contribution to protein digestibility by the chick pancreas acetone powder is very small and has been measured by us to be not more than 1 to 2%, it does remove the starch, which is essential for carrying out the workup described and, if desired, for estimating dry matter digestibilities (DMD).[3] In addition, the acetone powder probably contains lipase, which would assist in determining DMD.

With the same wheat millfeeds as substrate, the pronase-chick pancreas acetone powder method has been compared with three other published procedures for determining protein digestibility. The method of Booth and Moran (1) was originally devised to measure protein digestibility in wheat bran, but suffers from a cumbersome experimental procedure. Chick et al. (2) also measured protein digestibility in wheat bran, but the procedure is difficult to duplicate because of inadequate description of conditions, etc. The method of Akeson and Stahmann (5) is useful, but in our experience those steps involving neutralization of incubation mixes vary with different substrates and so result in a somewhat more cumbersome and lengthy procedure. The actual values are shown in Table III. In general, the figures for protein digestibility were slightly higher with these previous systems.

An advantage of using pronase is that this enzyme degrades protein to amino acids. Zimmerman[4] has used pronase to digest cereal-grain protein, and by direct evaluation of the amino acids produced has been able to assess a nutritive value for the protein.

One series of experiments where the pronase-chick pancreas acetone powder method has been successfully applied is in work described by Miladi et al. (13). These workers measured the relative nutritive value (RNV) of wheat-millfeed protein by a rat-feeding technique. Excellent correlation was obtained between RNV (as measured by growth response) and "digestible" lysine as measured by this *in vitro* procedure in the millfeeds tested.

Protein-digestibility figures for wheat millfeeds measured here agree generally with values found by other workers, both *in vivo* and *in vitro*. Summers et al. (12)

[3]Chick pancreas α-amylase can be satisfactorily replaced by other starch-hydrolyzing enzymes, though the workup is technically more difficult, apparently owing to fatty material preventing good centrifugation.

[4]G. Zimmerman, PL-480 Grant Project: A 10-AMS-7(a) (1966).

found in rat-feeding experiments values of 59.6 to 64.5% for brans, 68.4 to 75.9% for shorts, 80.0 to 87.5% for red dog, and 79.5 to 85.6% for germ. Olsen et al. (14), with rats, found values of 72.8 and 76.0% for bran, and 79.0 to 85.5% for shorts. Earlier workers had found (*in vivo*) for bran values of 63% (2) and 70 to 75% (15). In the earlier *in vitro* studies, Chick et al. (2) found a value of 74% for bran, Booth and Moran (1) found 72%, and Rohrlich and Rasmus (3) found 59 and 72%. It is obvious that there is quite a variation within each species. We believe this is due mainly to the variation in endosperm content carried through with the bran during the milling process, and in part to variation in wheat species and in the actual age of the millfeed. *In vitro* data in our laboratory indicate a very slow but gradual decrease in protein digestibility with age in wheat bran. Protein digestibility in wheat millfeeds has previously been shown to decrease with increasing storage time (16).

The amino acid pattern of the indigestible protein fragment prevalent in bran after *in vitro* or *in vivo* (fecal residue from chicks fed bran) digestions and the amino acid pattern of the undigested bran protein are listed in Table IV. The patterns of the digested products are very similar. The same phenomenon has been shown to be true for shorts (17).

It is likely that the protein in the branny layers is of limited digestibility in wheat millfeeds. As the amount of these layers in the millfeeds decreases (bran>shorts>germ>red dog>flour), there is a corresponding increase in the amount of protein which is available. The indigestible protein can probably be classified into two types. The first type of protein actually resides in the aleurone layer and is of limited digestibility because of the thick cell-wall matrix interfering with digestion or the protein is tightly bound to the cellulosic matrix of the aleurone cells. This phenomenon is indicated by the fact that *in vitro* protein-digestibility measurements on wheat bran pretreated with cellulase (which hydrolyzes the aleurone cell wall) showed values near 90%. The second type of protein (i.e., the remaining 10%) remains indigestible even after severe cellulase treatment[5].

TABLE IV. AMINO ACID PATTERN OF PROTEIN IN WHEAT BRAN, AND PROTEIN REMAINING UNDIGESTED AFTER IN VIVO OR IN VITRO DIGESTION OF THE WHEAT BRAN

Amino Acid g. AA/16 g. N	Whole Bran	Digested Bran In Vitro	Digested Bran In Vivo	Amino Acid g. AA/16 g. N	Whole Bran	Digested Bran In Vitro	Digested Bran In Vivo
Lysine	4.1	4.1	4.0	Alanine	4.8	5.4	5.2
Histidine	2.6	2.8	2.5	Half cystine	1.1	0.5	0.4
Ammonia	2.6	1.9	1.9	Valine	5.2	5.8	5.1
Arginine	6.9	7.8	8.0	Methionine	1.7	0.9	1.2
Aspartic acid	7.1	7.7	7.3	Isoleucine	3.7	3.7	3.4
Threonine	3.2	3.7	3.6	Leucine	5.9	6.5	6.1
Serine	4.3	4.5	4.4	Tyrosine	2.6	2.6	2.5
Glutamic acid	18.4	10.8	11.3	Phenylalanine	3.9	3.8	3.8
Proline	5.5	4.3	4.1	% Nitrogen			
Glycine	5.7	6.9	6.4	recovered	87.9	85.0	82.0

[5]R. M. Saunders, M. A. Connor, R. H. Edwards, and G. O. Kohler, unpublished work.

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