Semiautomated in Vitro Analysis of Sorghum Protein Availability via Pronase Hydrolysis[1]

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ABSTRACT

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Sorghum varieties with varying kernel structures were analyzed for availability of protein. Small (100-mg) samples of ground grain were digested with a protease enzyme, pronase, and optimum pH, time, and temperature. The hydrolysate was assayed for α-amino groups by monitoring absorbance at 570 nm following reaction with ninhydrin. Pronase digestibility was determined for 196 sorghum samples, including those without a testa ($b_1b_1b_2b_2ss$), with a testa (B_1-B_2-ss), with a testa and a spreader gene (B_1-B_2-S-), and with waxy (wxwxwx) and nonwaxy (WxWxWx) endosperm types. Sorghums with a testa showed significantly lower in vitro digestibility than sorghums without a testa. Sorghums with waxy endosperm had slightly higher digestibility than did nonwaxy sorghums. Pronase digestibilities of 11 sorghum samples were compared with those obtained by a multienzyme in vitro digestibility method, which grouped sorghums together in a narrow range of digestibilities. The pronase method was more sensitive, separating sorghums into groups according to observable characteristics.

Characteristics often used to define protein quality of a feed or food are its amino acid composition and its protein digestibility. Protein digestibility primarily determines the availability of its amino acids. Historically, protein digestibility has been determined by bioassays using rats or microorganisms. These procedures share the disadvantages of being time-consuming and expensive.

Several in vitro procedures to measure protein digestibility exist. These include both single and multiple enzyme systems. Multiple enzyme systems have shown better correlation with in vivo data than have systems utilizing only a single enzyme. A multiple enzyme system may reduce the effects of endogenous inhibitors specific for a single enzyme. Such a system also reduces the effect of variations in amino acid composition from sample to sample.

Hsu et al (1977) estimated protein digestibility in less than 1 hr using a multiple enzyme system consisting of trypsin, chymotrypsin, and peptidase. The system was repeatable and sensitive enough to detect changes in digestibility due to processing of a specific food product. Pepsin-pancreatin (Akeson and Stahmann 1964) and pepsin-trypsin (Saunders et al 1973) enzyme systems showed high correlations with in vivo data (0.87 and 0.91, respectively), whereas a pepsin system (Buchanan and Byers 1969) showed poor correlation. These methods are either complicated and time-consuming, involving multiple digestions and washings, or as with the Hsu et al (1977) method, not adaptable to automation.

The purpose of the present study was to develop an in vitro assay of protein digestibility that was simple, capable of being automated, and adequately sensitive to detect differences in digestibility among sorghum genotypes.

Use of pronase as the digesting enzyme in such an assay has several advantages over use of other enzymes or enzyme combinations. In the past, pronase has been used to isolate double-stranded deoxyribonucleic acid from protein (Pfau and McCrea 1962), to digest almost any protein virtually to free amino acids (Nomoto et al 1960), and to solubilize sorghum endosperm proteins (Sullins and Rooney 1974). Pronase shows no sequence specificity for hydrolysis and releases amino acids from both the carboxyl and amino terminals of peptides (Barker 1971). As a result, it can hydrolyze all available protein into component amino acids and/or small peptides, thus giving a true index of the total digestibility of the protein in the sample. The use of a single enzyme system reduces the variability induced by weighing errors. It also reduces the cost. The current cost for enzymes and reagents for the multienzyme method of Hsu et al (1977) is $1.17 per sample and for the pronase method, $0.51. Finally, pronase does not denature easily and therefore can be prepared and handled without undue loss of activity.

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MATERIALS AND METHODS

Grain Samples

One hundred ninety-six sorghum samples that varied in endosperm type, endosperm texture, presence or absence of a pigmented testa, and pericarp color were used in this study. These samples were obtained from plantings at Lubbock, TX, in 1971, 1972, 1978, and 1979 and from Hyderabad, India, in 1979. The 1978 and 1979 Lubbock samples were from panicles taken from the same row, segregating as waxy (wxwxwx) or nonwaxy (Wx----). All samples were stored as cleaned, sound grain at −0.4° C until used.

Enzymes

Pronase (*Streptomyces griseus* protease), B grade, with an activity of 45,000 proteolytic units per gram was obtained from Calbiochem-Behring Corporation. A proteolytic unit is defined as the number of μmoles of substrate converted per minute at 30° C by 1 mg of enzyme preparation. Peptidase, α-chymotrypsin, and trypsin were the same as those used by Hsu et al (1977).

Chemicals

Standard Animal Nutrition Research Council casein was obtained from Nutritional Biochemicals, Cleveland, OH. All chemicals were reagent grade.

In Vitro Digestibility with Pronase

Each sample of whole grain was ground in a Udy laboratory mill to pass through a screen 1.0 mm in diameter. The digestion procedure was modified from that of Lamar (1973). Casein was included as a standard in each series (or set) of digestions. For each sorghum variety, triplicate samples of 0.100 g were weighed into separate screw-top centrifuge tubes, and 20 ml of enzyme solution (2 mg of pronase per milliliter in 0.2*M* citrate-phosphate buffer at pH 7.0) was added. The final enzyme concentration was 18 units per milligram of sample. Preliminary studies (Fig. 1) relating concentration of enzyme in the digestion mixture to micrograms of amino nitrogen released per milligram of protein demonstrate that this concentration lies on that portion of the curve least subject to variations due to changes in enzyme concentration (slope approaching 0). Sample plus enzyme were incubated in a shaking water bath at 40° C for 4 hr. Proteolysis was stopped by the addition of 0.5 ml of 50% trichloracetic acid. After the particulate matter settled out, an aliquot of the supernatant was removed and assayed for free amino groups released during digestion. Free amino groups

were quantified by reaction with ninhydrin in the automated system described below. In all cases, appropriate blanks were included to correct for pronase autolysis and for endogenous free amino groups.

In vitro digestibility was recorded as micrograms of amino nitrogen per milligram of Kjeldahl nitrogen (AACC 1976). Amino nitrogen was determined as leucine equivalents by the ninhydrin assay. Other than those released by the action of pronase, no significant amounts of ninhydrin-positive compounds were measurable in ground whole sorghum.

Automated Ninhydrin Assay

The analytical system consisted of a Technicon Auto-Analyzer sampler, a pump, a 92°C oil bath, and spectrometric and recorder modules. Figure 2 is a flow diagram of the system. Up to 60 samples per hour were run with good resolution. The procedure is an adaptation of methods of Broderick (1978) and Gauger and White (1970). Hydrindantin, the compound reacting with the α-amino nitrogen to yield the colored product measured in the assay, is not present in the ninhydrin reagent. Therefore, the reagent is stable at room temperature in the presence of air. Hydrazine in the assay system reduced ninhydrin to the active hydrindantin. To prevent its breakdown, the system was operated under nitrogen.

Ninhydrin Reagent

Ninhydrin (6.67 g) was dissolved in 500 ml of ethylene glycol monomethyl ether. To this was added 100 ml of 4*N* sodium acetate buffer (pH 5.5) and 28.33 ml of glacial acetic acid. Final dilution was to 1 L with distilled water. The solution was allowed to stand overnight (8–12 hr) before use and was stored in an amber bottle open to air. Fresh reagent was made at least monthly.

Hydrazine Sulfate Reagent

Hydrazine, 0.25 g, was dissolved in 1 L of distilled water and acidified with one or two drops of concentrated sulfuric acid. Reagent was stored in a borosilicate glass bottle. Fresh reagent was made at least weekly.

Multienzyme in Vitro Digestibility

The in vitro method of Hsu et al (1977) was used. This technique is based on a multienzyme digestion system consisting of trypsin, chymotrypsin, and peptidase. It measures the drop in pH over a 10-min interval due to the release of ionizable carboxyl groups during proteolysis. Protein digestibility (Y) was calculated by the following regression equation from the pH after 10 min.

$$Y = 210.45 - 18.10\,(x)$$

Enzyme-Susceptible Starch Assay

Both total starch and enzyme-susceptible starch were quantitated by their susceptibility to glucoamylase attack, following the method of Akingbala et al (1981). Diazyme L100 (Miles Laboratories, Inc., Elkhart, IN) was used as digesting enzyme. Glucose released by hydrolysis was determined using Technicon method SF4-0045 FA8 (Technicon 1978), and results were reported as milligrams of glucose released per gram of starch.

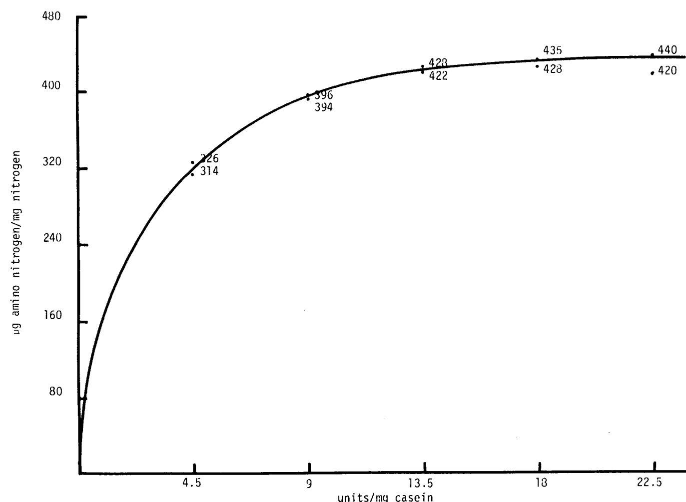

Fig. 1. Plot of enzyme concentration vs protein hydrolysis.

RESULTS AND DISCUSSION

Twenty-five sorghum samples grown at Hyderabad, India, were digested in triplicate on two separate days to identify and correct potential sources of error in the pronase digestion procedure. Casein samples were included in each day's analyses. Analysis of variance was performed on the resulting data (Table I). A significant day effect in the method was found, most likely introduced by weighing and dispensing errors during the preparation of the enzyme solution. Fresh preparation for each digest may introduce variation in enzyme concentration and activity from digest to digest. This was corrected by the inclusion of enzyme blanks and the use of casein standards. Digestion values for casein standards can be adjusted to a common mean and an adjustment factor determined for comparison of samples from different enzyme digests. No adjustment was necessary for samples in the same digest because no significant sample effect occurred (Table I). The use of a casein standard reduced the coefficient of variation from 18.23% to an acceptable 6.08%.

Sorghums of differing pericarp and testa structures (Types I, II, and III) and endosperm composition (waxy and nonwaxy) were selected and their in vitro digestibilities determined. The mean pronase digestibilities of casein, of sorghum Types I–III, and of waxy and nonwaxy sorghums are shown in Fig. 3. Sorghums had been divided into three groups or types based on the genes controlling seed color (Rooney et al 1980): Type I, sorghums

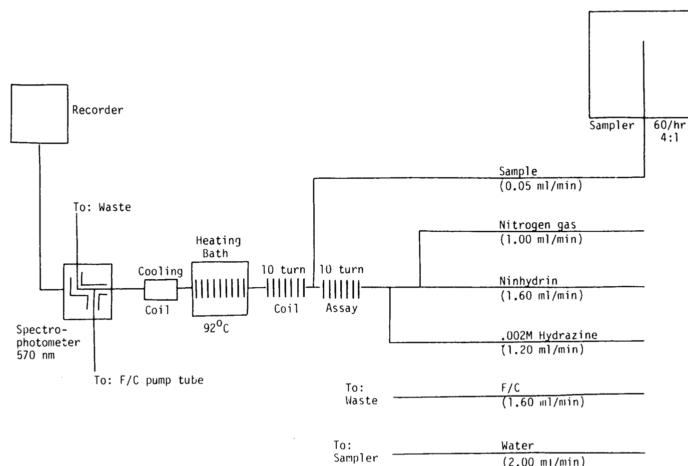

Fig. 2. Flow diagram for the automated ninhydrin assay. FC = flow cell.

TABLE I
Analysis of Variance[a] of Pronase in Vitro Digestibility of 25 Samples Grown in India in 1979

Source of Variation	Degrees of Freedom	Mean Sum of Squares[b]
Varieties (V)	22	8473.04**
Days (D)	1	5039.23**
Samples	2	196.74
V × D	22	934.98**
Error	82	318.30
Total	128	1856.06

[a]Standard deviation = 17.84, mean = 294, standard error of the mean = 3.79, coefficient of variation = 6.08.
[b]** = Significant at 0.01.

without a testa ($b_1b_1B_2$---) or (B_1-b_2b_2--); Type II, sorghums with a testa but without the spreader gene (B_1-B_2-ss); and Type III, sorghums with both a testa and a spreader gene (B_1-B_2-S-). When mean protein digestibilities were analyzed by Duncan's multiple range test, Type I sorghums showed significantly higher digestibilities than Types II and III. Also, Type II sorghums had higher digestibilities than Type III. Polyphenolic compounds increased from Type I to Type II to Type III sorghums (Earp et al 1981). Polyphenols bind to and precipitate protein (Butler et al 1979), reducing protein availability. The level of polyphenolic compounds, as measured by the modified vanillin method (Maxson and Rooney 1972), was negatively correlated with pronase in vitro digestibility (−0.83). This corresponds to the reduced digestibility of Type II and III sorghums.

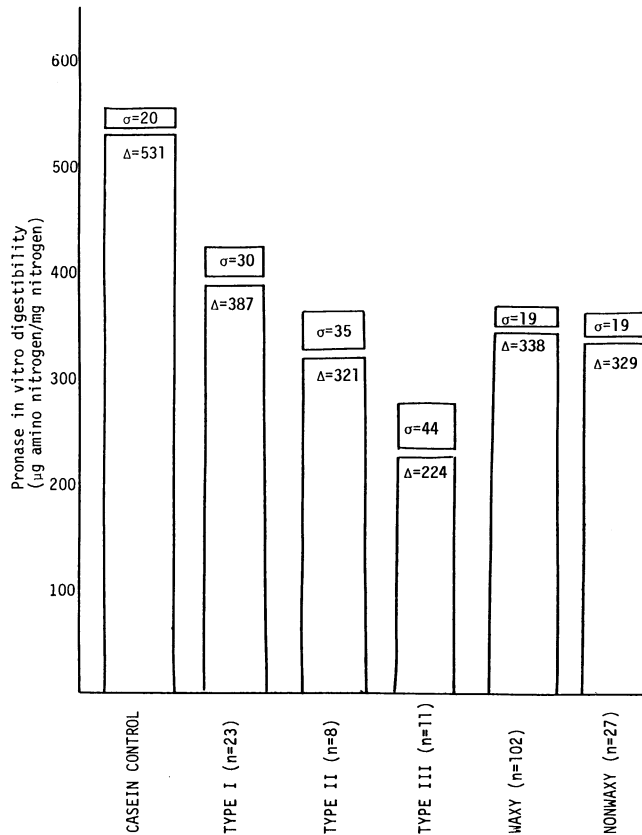

Fig. 3. Pronase digestibility for casein and for Type I, Type II, Type III, waxy, and nonwaxy sorghums.

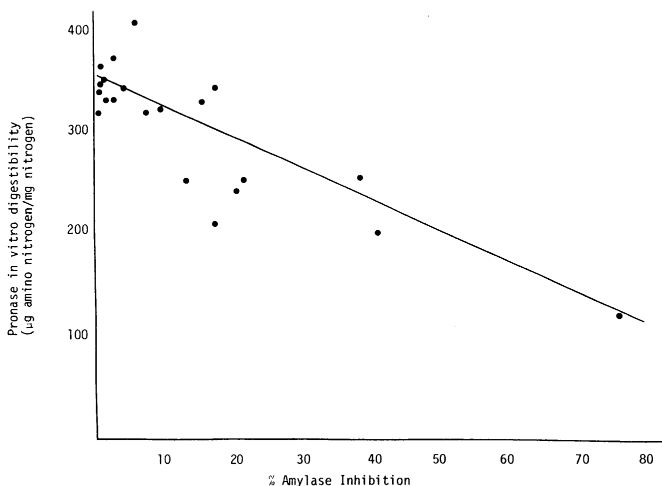

Fig. 4. Pronase in vitro digestibilities and percent inhibition of amylase for 21 sorghums.

Protein binding and precipitation by polyphenolic compounds is the basis of one method for tannin quantitation (Davis and Hoseney 1979). In this method, tannins are measured by their ability to reduce the activity of α-amylase in solution. The reduced digestibilities of Types II and III grain stimulated studies to test the possibility that enzyme inhibition and relative digestibility might share a common mechanism, ie, polyphenol binding to enzyme and/or substrate. Inhibition of α-amylase by 21 sorghum samples grown at Lubbock, TX, in 1971 and 1972 was determined by Earp et al (1981), using the method of Davis and Hoseney (1979). Figure 4 shows the percent amylase inhibition plotted against the pronase in vitro digestibility. The negative correlation (−0.85) suggests that a similar inhibitory mechanism is active in both cases.

Waxy sorghums tended to have higher pronase digestibility than did nonwaxy sorghums. The difference was not great enough to conclude that waxy grain has a higher digestibility than nonwaxy grain. A comparison of pronase digestibility, enzyme-susceptible starch, and 1,000-kernel weight of 14 waxy and nonwaxy samples grown at Lubbock in 1979 is shown in Fig. 5. A positive value

Fig. 5. Comparison of waxy vs nonwaxy sorghums from a segregating population grown in 1979 at Lubbock, TX.

indicates that the waxy grain had a larger value than its nonwaxy counterpart. The samples were taken from the same rows of segregating populations, in which the only difference was the presence or absence of the waxy gene. The waxy grain showed an increased starch digestibility compared to that of the nonwaxy grain in all 14 comparisons. In 11 of the 14 samples, waxy grain showed higher protein digestibility than that of its nonwaxy counterparts. However, only four of the 11 were significantly different.

Waxy grain had lighter kernels with higher starch and protein digestibilities. However, in four comparisons, the waxy had equal or heavier kernels and increased protein and starch digestibilities. The existence of sorghums with these characteristics indicates that development of waxy varieties with improved starch and protein digestibilities coupled with kernel size and yield characteristics equivalent to those of nonwaxy sorghums may be possible. Sullins and Rooney (1974, 1975) observed that enzyme susceptibility of both the starch and protein components was higher in waxy than in nonwaxy grain. Waxy sorghums have been shown to have improved feed efficiency and digestibility in steers (McCollough 1973, Sherrod et al 1969), sheep (Nishimuta et al 1969), and finishing pigs (Purser 1979). The known differences between waxy and nonwaxy genotypes lie in the composition of their starches, ie, the ratio of amylose to amylopectic, and an apparent alteration in the way protein is distributed in the kernel, especially in the peripheral or subaleurone endosperm (Sullins and Rooney 1975). Whether these differences result from an alteration in amino acid sequence, composition of a particular protein fraction, or strength of the protein starch interaction is unknown, but they may account for the greater digestibility of the waxy endosperm.

In an attempt to compare the pronase method with current in vitro digestibility tests, the digestibilities of 11 sorghum samples were determined by both the pronase method and the multienzyme method (Hsu et al 1977) (Fig. 6). The multienzyme method grouped most sorghums into a narrow range of digestibilities. The pronase method was more sensitive, capable of grouping sorghums by their kernel characteristics. The correlation coefficient between the methods was 0.76.

The major difference between the two methods is the manner in which the digestibility is quantified. The pronase method measures the release of amino groups, whereas the method of Hsu et al (1977) measures the drop in pH due to the release of ionizable carboxyl groups. The sorghum flours used in both digestions contain a relatively low protein level (~10%) compared to levels of other components. The high solids level in the Hsu et al (1977) method

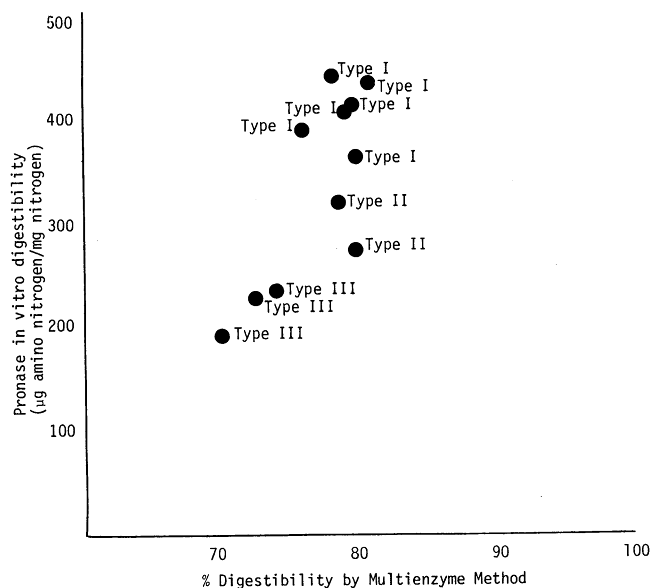

Fig. 6. Comparison of the digestibilities of 11 sorghum samples determined by pronase and multienzyme (Hsu et al 1977) in vitro methods.

(~60 mg/ml) is difficult to maintain as a homogenous suspension. Inadequate mixing may cause erroneous results due to nonuniform contact of suspension with measuring electrode or of enzyme with substrate. Pentosans, phenolic acids, and other ionizable compounds may act to buffer the system and reduce the sensitivity of the Hsu et al method.

The use of pronase in a partially automated assay for in vitro protein digestibility is promising. In the analysis of 196 sorghum samples, the method was sensitive enough to demonstrate differences in digestibility among sorghums of varying kernel structures and compositions. Use of a single enzyme and automation of the final analytical steps make the procedure capable of handling large numbers of samples economically.

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