are likely. Cooking sheep meats have been shown to evolve hydrogen sulfide in greater quantities, and at a greater rate, than beef (Kunsman and Riley, 1975). In view of the low odor thresholds of these sulfur-containing compounds (Shankaranarayana et al., 1974; Schutte, 1974), the quantitative differences in these and other, highly odorous sulfur-containing compounds may be of importance in the distinctive species odors.

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Supplementary Material Available: A table of mass spectral details of indicated compounds (2 pages). Ordering information is given on any current masthead page.

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Protease Activity of Water- and Acid-Reconstituted Grain Sorghum

Richard E. Lichtenwalner,* George I. Glover, and Ching Chia Shaw

Protease activity of grain sorghum as affected by variety, reconstitution with water, and reconstitution with propionic acid was determined. Varieties having smaller kernel size had higher levels of protease activity than a similar variety with larger size kernels. Protease activity was highest on dry grain and declined during anaerobic reconstitution. Reconstitution with 2% w/w propionic acid accelerated the rate of decline of protease activity. Protease activity increased during aerobic reconstitution. The level of soluble nitrogen in propionic acid reconstituted sorghum was greater ($P < 0.05$) than water-reconstituted grain. This increase in protein solubility of propionic acid reconstituted grains indicates that either initial levels of protease are adequate or that solubilization of proteins is due to factors other than proteases in grain.

Sorghum is the fourth leading cereal grain produced in the world and serves as a major feed constitutent for livestock. Although comparable in nutrient content to other feed grains, digestibility coefficients of sorghum nutrients are generally lower (NRC, 1970). The reduced availability of sorghum nutrients may be due to the physical arrangement of nutrients within the kernel. In the peripheral endosperm of sorghum, the starch granules are embedded in a protein matrix that is poorly digested by ruminants (Sullins et al., 1971; Walker and Lichtenwalner, 1977). This structural arrangement of starch granules and protein is under genetic control. In nonwaxy or normal sorghum, the peripheral endosperm starch granules are small and tightly packed in a proteinaceous matrix. In the genetic mutant, waxy sorghum, the starch granules are larger and less tightly packed (Sullins and Rooney, 1974). This may account for the increased digestibility of waxy sorghums.

Processing of sorghum improves nutrient availability and reconstitution consumes less energy than heat processing (Lipper et al., 1976). Reconstitution is the process of adding sufficient moisture back to grain and sealing the moist grains from the environment so fermentation can occur. In some instances, propionic acid is added to the grain at time of reconstitution to deter fungal growth. Sullins et al. (1971) noted in a histological study that the protein matrix of reconstituted grains was partially disrupted. This is consistent with the earlier proposal that degradation of the protein matrix accounts for the increased feed efficiency of cattle fed reconstituted grain

Department of Animal Science, Texas A&M University, College Station, Texas 77843.

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(Florence et al., 1968). The disruption could be the result of physical swelling of the grain and/or of partial enzymatic degradation of the protein in the matrix. The latter possibility is attractive since proteases exist in the grain to break down the matrix to facilitate starch utilization during germination (Mounfield, 1936). Garg and Virupaksha (1970) have shown that the amount of acid protease, with the unusual specificity for cleavage of peptide bonds at acidic amino acid residues, increases rapidly in the endosperm when seeds are germinated on moist filter paper. Ingle et al. (1964) described the physical and chemical changes taking place in grain during germination and reported that they appeared to be similar to those occurring during reconstitution. The purpose of this research was to determine the level of protease activity during water or acid reconstitution in several varieties of sorghum grains.

MATERIALS AND METHODS

Experiment 1. Sorghum grains used for these experiments were P.A.G. (a commercially available hetero-yellow), SC301 (a corneous endosperm), Redlan Waxy (B-413), and Redlan Nonwaxy (TX378). The Redlan grains were near isogenic, differing mainly in the gene responsible for the waxy characteristic.

Moisture content of the grains was determined prior to reconstitution by drying the sample to a constant weight at 85 °C in a vacuum oven. Due to the difference in kernel size between Redlan waxy and nonwaxy sorghum, protease activity was also measured on these two lines on the basis of an equal number of kernels. Thirty, 60, 90, and 120 air-dry kernels of each line were assayed for protease activity as described below.

Reconstitution was accomplished by adding sufficient deionized water to 4 g of air-dry whole grain to bring the moisture level to 30%. The grains were then stored in closed glass vials at room temperature. During reconstitution, protease activity was measured at the intervals indicated in the figures.

Extracts of protease from 4 g of reconstituted or dry seeds were obtained by homogenizing the seeds with 9 mL of 0.05 M sodium phosphate buffer, pH 7.0, in a Waring blender for 1 min at high speed and 2 min at low speed and allowing the mixture to stand for 1 h. The homogenates were centrifuged at 12000*g* for 10 min. The supernatant was dialyzed against 10 volumes of buffer for 8 h. The dialysis procedure was repeated three times.

Proteolytic activity was determined similar to the method of Garg and Virupaksha (1970). Dialyzed enzyme extract (0.5 mL) was added to 2.0 mL of bovine serum albumin (Sigma Chemical Co.) solution (5 mg in 0.05 M sodium citrate buffer, pH 3.5). After 50 min at 40 °C in a water bath, the reaction was terminated by adding 2.0 mL of 10% trichloroacetic acid to the incubation mixture, heating the mixture briefly in boiling water, and then centrifuging the precipitate of undigested albumin. To 0.625 mL of supernatant, 1.25 mL of 0.5 N sodium hydroxide was added, followed by the addition of 0.375 mL of 1.0 N Folin-Ciocalteau reagent (Sigma Chemical Co.). After mixing (vortex) and allowing the reaction to stand for 30 min for color development, the concentration of hydrolyzed protein in the acid supernatant was determined by reading the absorbance at 660 nm against a blank in which the substrate, enzyme extract, and 10% trichloroacetic acid were added at the same time intervals. A control using the protease activity of pepsin (Sigma Chemical Co.) was included in each experiment to insure that the assay was working properly. The points on each figure representing protease activity were the average of six determinations run simultaneously on six different 4-g samples.

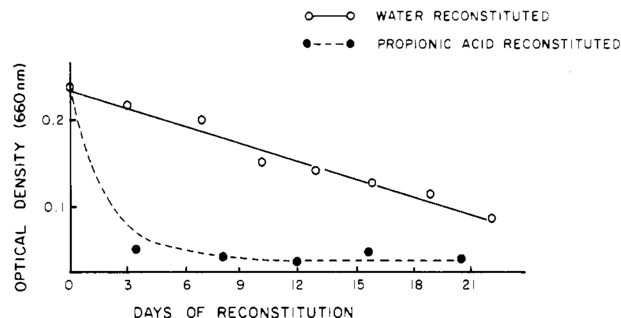

Figure 1. Protease activity of P.A.G. sorghum during reconstitution.

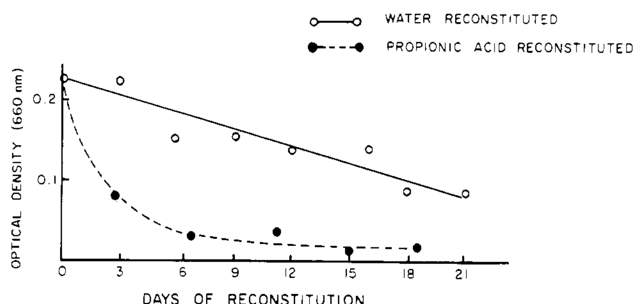

Figure 2. Protease activity of SC301 during reconstitution.

Experiment 2. The effect of propionic acid on protease activity during reconstitution was determined by adding propionic acid to the reconstitution media at a level of 2% (w/w). The procedures for the extraction and assay of protease were similar to those of experiment 1 with the exception that, during extraction, the kernels from the propionic acid treated grains were suspended in 4 mL of phosphate buffer prior to blending, and the pH was adjusted to 7.0 with sodium hydroxide. This was done to compensate for the pH effect of acid on the extraction of the protease. In addition, the effect of propionic acid reconstitution of Redlan waxy grains was not determined.

Nitrogen content was determined on the whole P.A.G. grain and on residues from homogenization (experiment 2) by Kjeldahl digestion, followed by colorimetric analyses (Crooke and Simpson, 1971). Percent soluble nitrogen was calculated by difference between levels of nitrogen in initial grain and nitrogen in residue. Difference between means was statistically treated by analysis of variance (Li, 1964).

Experiment 3. The effect of oxygen on protease activity during reconstitution was determined. Four grams of SC301 sorghum were reconstituted to a final moisture content of 30%. Aerobic conditions during reconstitution were achieved by loosely placing the grain in open glass vials and storing for 0, 2, and 4 days. Anaerobic reconstitution and protease activity analysis were accomplished as in experiment 1.

RESULTS

Experiment 1. Protease activity of all lines of sorghum investigated was greatest in air-dry seeds and steadily declined during reconstitution (Figures 1–3). After 21 days, protease activity of water reconstituted grain was approximately 30% of original level. The lower protease activity of Redlan nonwaxy (Figure 3) is apparently due to a difference in kernel size. Four grams of air-dry Redlan waxy contained 131 kernels but only 109 kernels of Redlan nonwaxy. When protease activity was determined on an equal number of air-dry kernels (Figure 4), no statistical differences were detected.

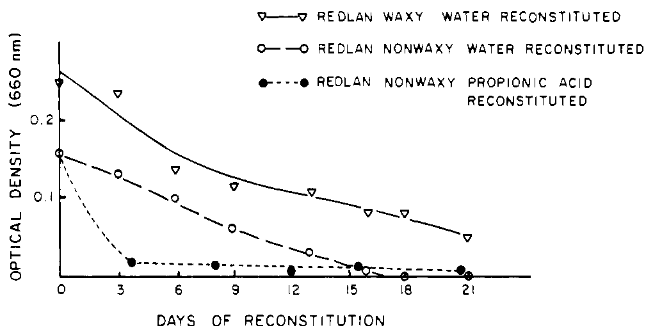

Figure 3. Protease activity of Redlan waxy and Redlan nonwaxy during reconstitution (equal weight samples).

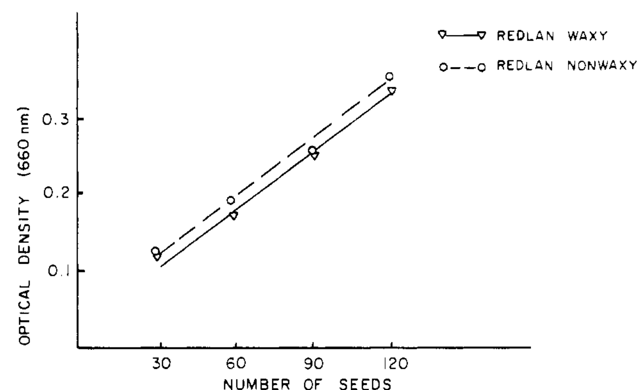

Figure 4. Protease activity by number of Redlan waxy and Redlan nonwaxy seeds.

Experiment 2. The effect of propionic acid on protease activity during reconstitution is also shown on Figures 1–3. After 3 days of reconstitution, protease activity of propionic acid treated kernels was less than 10% of original levels. Protease activity in propionic acid reconstituted kernels was not affected by variety.

Nitrogen content of the grains, homogenized residue, and supernatants is shown in Table I. Mean level of soluble nitrogen was higher in the propionic acid reconstituted grain than original grain ($P < 0.01$) or water-reconstituted grain ($P < 0.05$). Level of soluble nitrogen remained fairly constant over time in the water-reconstituted grain, but tended to increase in the propionic acid reconstituted grain.

Experiment 3. Protease activity of aerobically and anaerobically reconstituted grain is shown in Figure 5. Aerobic reconstitution for periods longer than 4 days resulted in excessive spoilage of the grain. It was uncertain whether the protease activity measured in these samples was of grain or fungal origin; therefore, the data are not presented. However, prior to visual observation of fungal invasion, protease activity of aerobically reconstituted grain increased with time, whereas protease activity of anaerobically reconstituted grain decreased.

DISCUSSION

The decrease in protease activity of grains during reconstitution has not previously been reported. Garg and Virupaksha (1970) demonstrated an increase in protease activity in germinated sorghum. The process of germination is aerobic, whereas reconstitution is anaerobic. When sorghum was reconstituted in the presence of air, protease activity increased (Figure 5). Thus it appears that the biochemical pathway(s) of protease is oxygen dependent and would function only in the early stages of the reconstitution process. The decrease in protease activity with time of reconstitution may reflect the decreasing supply of oxygen in the reconstituted grain mass. However, since microbial fermentation also increases as oxygen diminishes, the decrease could also be due to degradation of the enzyme by microorganisms.

Table I. Effect of Time and Propionic Acid on Nitrogen Solubility of Reconstituted Sorghum

grain treatment	days of reconstitution	total nitrogen, %	soluble nitrogen, % of total
dry		2.03	16.0
water reconstituted	4		21.8[a]
	8		20.1[a]
	12		21.7[a]
	16		18.3
	20		19.3[a]
			20.2[a]
propionic acid reconstituted	4	$\bar{X}$	21.2[a]
	8		22.6[a]
	12		24.1[a]
	16		28.2[b]
	20		26.0[b]
		$\bar{X}$	24.4[b]

[a] Level of soluble nitrogen greater ($P < 0.05$) than dry grain. [b] Level of soluble nitrogen greater than dry grain ($P < 0.01$) or water reconstituted grain ($P < 0.05$).

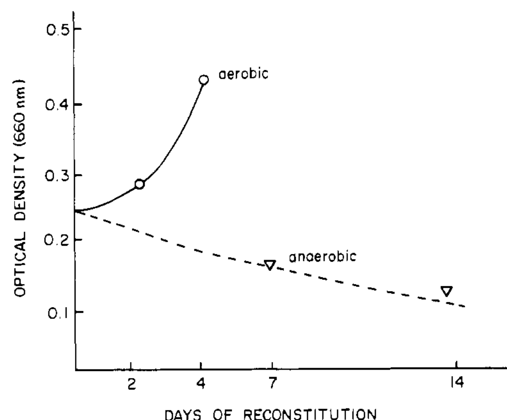

Figure 5. Protease activity of SC301 kernels during reconstitution under aerobic and anaerobic conditions.

The abolition of protease activity in propionic acid reconstituted grains was first thought to be an artifact due to pH differences. However, buffering the extraction media did not prevent the rapid decrease of protease activity. The mechanism by which propionic acid decreases protease activity is unknown. One possibility was that acid conditions enhance the reaction between tannins and proteins; however, the tannin content of all grains evaluated is very low (Maxson, 1973). Another possibility is that the pH of acid-reconstituted grain is lower than water-reconstituted grain (Prigge et al., 1976). However, the pH optimum of the protease enzyme is 3.6 (Garg and Virupaksha, 1970).

Varietal differences in protease activity of reconstituted sorghum were minor. Although the higher activity in Redlan waxy vs. nonwaxy may indicate greater levels or accessibility of the enzyme, the similarities in activity when compared on an equivalent number of seeds basis would indicate a physical difference. Since the enzymes originate in the aleurone layer (MacLeod and Palmer, 1966) in the pericarp of the kernel, smaller kernels would have proportionately more aleurone layer, and therefore enzyme, on the equivalent test weight basis of 4 g.

Theoretically, an increase in protease activity should result in an increase in soluble introgen. As indicated in

Table I, nitrogen solubility was greater ($P < 0.05$) in the propionic acid reconstituted grain which also had lower protease activity. Prigge (1976) also reported an increase in nitrogen solubility in acid-rather than water-reconstituted grain. This would suggest that either the initial level of protease activity is sufficient to alter chemical composition or other proteolytic enzymes are active during reconstitution. A large proportion of the increase in soluble nitrogen was achieved by the fourth day of reconstitution. A rapid increase in nitrogen solubility of reconstituted grain was also reported by Wilfong (1969). Therefore it may be that initial levels of protease activity are effective in solubilizing protein. Other sources of proteolytic activity exist in the microbial population that expands during the fermentation process of reconstitution. In this regard, fumigation of grain prior to aseptic reconstitution decreased nitrogen solubility (Billings, 1972). Thus, it would appear that several sources of proteolytic activity exist in reconstituted grain and that the protease activity of plant origin is not the sole enzyme responsible for chemical alterations of reconstituted sorghum.

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Distribution of Saponin in Alfalfa Protein Recovery Systems

A. Lyle Livingston,* Benny E. Knuckles, Richard H. Edwards, Donald deFremery, Raymond E. Miller, and George O. Kohler

Saponin tended to be concentrated in the protein fractions at levels higher than in the original alfalfa during the preparation of white and green alfalfa leaf protein fractions. Coagulation of the protein at pH 6.0 and washing at pH 4.5 gave as much as a fourfold increase in saponin over that in the protein coagulated and washed at pH 8.5. The press cakes from high and low saponin alfalfa, following dejuicing, retained 65 and 87% of the saponin, respectively. The green protein contained most of the extracted saponin. Preparation of protein fractions from a low saponin cultivar resulted in saponin levels of less than 0.07%, compared to a saponin content of 1.33% in the protein prepared from a high saponin alfalfa cultivar. Six common vegetable food products were found to contain levels of saponin (0.02 to 0.07%) similar to that found in the white leaf protein fraction.

The high production of protein in alfalfa plants offers a source of nutrients which can help meet the protein requirements for humans as well as animals. The preparation of both whole and fractionated alfalfa leaf protein concentrates (LPC) has been studied for many years by workers such as Pirie (1966) in England and by Stahmann (1968) at the University of Wisconsin and more recently by workers from this laboratory (Edwards et al., 1975; and Kohler and Knuckles, 1977). Alfalfa LPC preparations have been shown to be excellent sources of nutrients for monogastric animals (Cheeke, 1974; Cheeke et al., 1977a; Cheeke and Myer, 1973; Kuzmicky and Kohler, 1977; Kuzmicky et al., 1977) and potentially for human consumption (Pirie, 1966; Bickoff et al., 1975). The possible contamination of these LPCs by naturally occurring biologically active compounds necessitates an investigation of the distribution of these compounds during the production of protein concentrates. Knuckles et al. (1976) determined the coumestrol content of alfalfa LPCs prepared under several processing conditions and observed that the protein concentrates, including both green LPC and white LPC, contained lower concentrations of the forage estrogen, coumestrol, than did fresh alfalfa or the resulting pressed alfalfa. In addition to the forage estrogens, alfalfa as well as other legumes have been found to be a source of biologically active saponins (Peterson, 1950; Walter et al., 1954; Birk et al., 1963) which may have a deleterious effect upon poultry (Tung et al., 1977) or other animals (Cheeke et al., 1977b). These alfalfa saponins are composed of long-chain carbohydrates attached principally to the aglycon, medicagenic acid (Djerassi et al., 1957; Gestetner, 1971) with smaller amounts of a second biologically active aglycon, hederagenin, also present (Shaney et al., 1972).

This study describes the distribution of the biologically active saponins during the preparation of LPCs by two different processes. In one process (Pro-Xan) the soluble and insoluble proteins are precipitated together (whole LPC) to provide a chlorophyll-pigmented-protein con-

U.S. Department of Agriculture, Western Regional Research Center, Science and Education Administration, Berkeley, California 94710.

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