Resistance of Sorghum α-, β-, and γ-Kafirins to Pepsin Digestion†

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The differences in pepsin digestibility among α-, β-, and γ-kafirins and the effect of treatment with sodium bisulfite were investigated in uncooked and cooked sorghum flour. Enzyme-linked immunosorbent assay revealed that when the untreated flour was incubated with pepsin, 31.0% of α-kafirin, 15.3% of β-kafirin, and 13.5% of γ-kafirin remained undigested. Transmission electron microscopy (TEM) showed that protein body digestion was initiated at the surface where β- and γ-kafirins are located. When the flour was treated with sodium bisulfite, the kafirins in the residue were much less and protein bodies were largely reduced in size. Cooking the flour reduced the digestibility of all three kafirins, particularly of the β and γ fractions. Some cooked protein bodies appeared unaffected by digestion as revealed by TEM. Sodium bisulfite reversed the effect of cooking, although not completely. It appears that on cooking, disulfide linkage formation is enhanced among β- and γ-kafirins, or among these and matrix proteins, which delays α-kafirin digestion. A reducing agent enhances the digestion of these cross-linked proteins and facilitates the exposure of α-kafirin to the enzyme.

Keywords: *Sorghum; protein; digestibility*

INTRODUCTION

Sorghum grain is the staple food for millions of people who live in the semiarid tropical regions of Africa, Asia, and Latin America. In many cases consumers of sorghum-based diets are quite dependent on available protein and energy from the grain. Human studies (Kurien et al., 1960; MacLean et al., 1981) have shown that protein from tannin-free sorghum porridge and Indian bread is poorly digested relative to other cereals (55 and 46% apparent digestibility, respectively). *In vitro* studies (Axtell et al., 1981; Hamaker et al., 1986) showed that cooking sorghum flour in water to a porridge decreases digestibility substantially. Some processing methods, such as fermentation and extrusion, increase digestibility (Graham et al., 1986; MacLean et al., 1983). Moreover, an *in vitro* digestibility study (Hamaker et al., 1987) showed that the use of reducing agents which cleave protein disulfide bonds increases digestibility significantly, nearly to the level of the other cereals in both uncooked and cooked sorghum flour. The underlying reason for the poor digestibility of unprocessed sorghum is not clear.

The protein bodies are the site of accumulation of kafirins, which are the aqueous alcohol-soluble storage proteins and the most abundant among the sorghum proteins. Kafirins have been classified into α (M_r 25 000 and 23 000), β (M_r 20 000, 18 000, and 16 000), and γ (M_r 28 000) on the basis of solubility, molecular weight, and structure (Shull et al., 1991). Kafirins are the last proteins to be digested in sorghum flour (Hamaker et al., 1986; Bach Knudsen and Munck, 1985), which is probably related in part to the composition and structure of the protein bodies in which they are located.

A scanning electron microscopy (SEM) study on pepsin digestion of sorghum protein bodies showed extensive pitting of uncooked protein bodies, while cooked protein bodies remained smooth in appearance and took longer to digest (Rom et al., 1992). Sorghum cooked with the reducing agent sodium bisulfite showed a pitting early in digestion similar to that of the uncooked protein bodies. These investigations support a previous postulate (Hamaker et al., 1987) that protein disulfide linkages form during cooking, thus impairing protein digestion, and that the addition of a reducing agent reverses or prevents this effect.

Immunocytochemical studies using polyclonal rabbit antibodies against the α-, β-, and γ-kafirins showed their differential location within the protein body (Shull et al., 1992). α-Kafirin, comprising about 80% of total kafirin, is located in light staining regions of the interior of the protein bodies, β-kafirin is found in dark staining areas inside and on the outside of the protein bodies, and γ-kafirin is located in the dark stained inclusion bodies of the inside as well as the dark staining areas on the outside. The β- and γ-kafirins contain unusually high proportions of the sulfur-containing residue cysteine (Shull et al., 1992). Because a large proportion of these two kafirins is extractable only in the presence of a reducing agent, they could be responsible for forming protein polymers resistant to digestion. We have recently found that unreduced, isolated α-kafirin is readily digested whether uncooked or cooked (B. R. Hamaker, unpublished data, 1993). Therefore, the difficulty in digesting α-kafirin may be due simply to its location inside the protein body.

A recent study on sorghum germination revealed that β- and γ-kafirins were the first proteins to be degraded (Mazhar and Chandrashekar, 1993). The authors concluded that because protein degradation occurs from the surface, the maximal breakdown of β- and γ-kafirins may be explained by their peripheral location. Germination studies in corn led to similar conclusions (Torrent et al., 1989).

Since α-, β-, and γ-kafirins seem to have distinctive features, it is critical to determine if they differ also in pepsin digestibility. The resistance to digestion of a specific protein(s) that would decrease the rate of

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Table 1. Percentage of Total Protein Extracted and of α-, β-, and γ-Kafirins Extracted after Various Treatments

treatment	extracted protein[a] (%)	extracted kafirins[b] (%)		
		α	β	γ
raw	92.9 (0.6)	100.0 (4.1)	100.0 (7.0)	100.0 (6.0)
cooked	88.6 (1.4)	95.0 (7.4)	91.9 (11.4)	88.1 (5.7)
cooked, sonicated	94.5 (0.5)	94.9 (9.1)	83.0 (9.9)	77.6 (3.0)
cooked, α-amylase	93.8 (4.5)	91.9 (5.3)	92.4 (4.5)	98.8 (2.9)
cooked, α-amylase, sonicated	101.5 (1.2)	100.0 (0.8)	93.7 (7.7)	91.7 (2.0)

[a] Protein extracted from the total amount. Mean from two determinations (SD). [b] α-, β-, and γ-kafirins extracted, 100.0% being the total extracted from the uncooked. Mean from two determinations (SD).

digestion of other proteins could be related to the low digestibility of sorghum protein. The objectives of this study were to investigate the susceptibility of the α-, β-, and γ-kafirins to *in vitro* pepsin digestion. Enzyme-linked immunosorbent assay (ELISA) was used to examine the digestibility of each kafirin fraction and how it is affected by cooking and addition of a reducing agent. Transmission electron microscopy (TEM) was used to observe the microstructural effects of each treatment in addition to examining how the location of kafirins within the protein body may affect digestibility.

EXPERIMENTAL METHODS

Plant Material. Sorghum (cv. P721N, 1988 and 1991 crop years) grown at Purdue University Agronomy Research Center in West Lafayette, IN, was used. Grain was collected at maturity and stored in a desiccator. Kernels for the protein study were decorticated for 2 min with a Strong-Scott barley pearler and ground for 15 s into flour with a Wig-L-Bug (Vivadent Dental Co. Inc., Amherst, NY). For the transmission microscopy study, whole grain was milled (Tecator Cyclotec 1093, Sweden) to pass through a 0.4 mm screen.

Uncooked Sample Preparation. Flour (200 mg) was weighed and either used directly for the *in vitro* protein digestion or soaked overnight in 5 mL of a 100 mM sodium bisulfite solution prior to pepsin digestion.

Cooked Sample Preparation. A preliminary study was done to establish a method that would extract the maximum amount of protein and kafirins from the cooked flour. Flour (200 mg) was cooked in water (5 mL) in a boiling water bath for 20 min, or it was cooked in a 0.08 M sodium phosphate buffer, pH 6 (5 mL), containing heat-stable bacterial α-amylase (50 μL) (Sigma Chemical Co., St. Louis, MO). After centrifuging, washing the pellet with deionized water, and again centrifuging, 6 mL of the extracting buffer [0.0125 M sodium borate buffer, pH 10, 2% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol (2-ME)] was added to the pellet, before the sample cooled. Then samples were either shaken for 16 h to extract the proteins or sonicated (cell disrupter Model B-12, Branson Sonic Power Co., Danbury, CT) for 4 min as described by Singh et al. (1990) before extraction of proteins. The nitrogen content of the extracts was determined according to micro-Kjeldahl Method 46-13 (American Association of Cereal Chemists, 1986). A 5.65 nitrogen to protein conversion factor (Mossé, 1990) was used. ELISA was used as described below to determine the amounts of α-, β-, and γ-kafirins extracted.

In view of the preliminary extraction results (Table 1), cooked samples were prepared as follows. Flour (200 mg) was cooked for 20 min in 5 mL of boiling 0.08 M sodium phosphate buffer, pH 6, containing 50 μL of heat-stable α-amylase. For the reduced samples, 200 mg of flour was cooked in a 100 mM sodium bisulfite solution of the same buffer (5 mL) containing α-amylase.

***In Vitro* Protein Digestibility Assay.** Uncooked and cooked flour samples (200 mg) were subjected to *in vitro* protein digestion as described by Mertz et al. (1984). Samples were incubated with pepsin solution (35 mL of 1.5 g of enzyme/L of 0.1 M potassium phosphate buffer, pH 2) for 2 h at 37 °C in a shaking water bath. Percent protein digestibility was calculated by subtracting undigested protein from total protein and dividing by the total protein. To determine the effect of gelatinized starch on protein digestibility, cooked flours treated with and without α-amylase were compared for protein digestibility.

Determination of α-, β-, and γ-Kafirins. ELISA was performed to determine the amounts of α-, β-, and γ-kafirins in the raw flour and, after digestion of proteins, in the indigestible residue. Uncooked and cooked samples were digested, with and without sodium bisulfite, as described before. Samples were washed three times with 0.08 M sodium phosphate buffer, pH 6. Nearly all of the protein was extracted from the undigested residues, as well as from an uncooked undigested sample (200 mg) that was used as a standard, by shaking with 6 mL of a solution containing 0.0125 M sodium borate buffer, pH 10, 2% SDS, and 1% 2-ME for 16 h at room temperature. All extracts were diluted 1250-fold in 40% (v/v) ethanol/10% (v/v) acetic acid, and α-kafirin assays were diluted another 5-fold in the same solvent. One hundred microliters of each diluted extract was added to the bottom of a microtiter plate (Immunolon 2, Dynatech Laboratories, Inc., Chantilly, VA) and diluted 2-fold. The samples were mixed, and six 2-fold dilutions were made in the plate. They were incubated at 30 °C for 2 h and rinsed three times with TBS-T (25 mM Tris-HCl, 0.9% NaCl, 0.05% Tween, pH 7.5). Then 100 μL of primary rabbit antibody (1:1000 in TBS-T) developed previously in this laboratory against α-, β-, and γ-kafirins (Shull et al., 1992) was added to the wells, and the plates were incubated for another 2 h at 30 °C. After three washes with TBS-T, the plates were left overnight at 4 °C, after addition of 100 μL of goat anti-rabbit alkaline phosphatase (Jackson Immunoresearch Laboratories, Westgrove, PA) in TBS-T (1:5000). Alkaline phosphatase substrate was prepared (Clark et al., 1986), and 100 μL of it was added to the wells, after three washes with TBS-T. After color development for 1 h at room temperature, absorbance at 405 nm was read using a Vmax ELISA reader (Molecular Devices, Menlo Park, CA). Absolute amounts of the different proteins were computed by dividing the slope of their linear regression by the slope of the line for the mature P721N standard and then multiplying by the milligram amount of each protein fraction per 30 mg of the standard. Tests for linearity and specificity for this ELISA are reported elsewhere (Oria et al., 1995).

Preparation of Microscopy Samples. Uncooked sorghum flour (200 mg) was used either directly or soaked overnight at 4 °C in 2 mL of a 100 mM sodium bisulfite solution. Cooked samples were prepared by suspending 200 mg of flour in 2 mL of water or 2 mL of 100 mM sodium bisulfite solution and boiled in a water bath for 20 min. All samples were subjected to *in vitro* pepsin digestion as described by Mertz et al. (1984). Control samples were sorghum flour treated according to the same methods as described above except that pepsin solution used in the protein digestion method was replaced with 0.1 M potassium phosphate buffer, pH 2. Following pepsin digestion, samples were sequentially digested with amyloglucosidase (*Rhizopus*, EC 3.2.1.3, 12 000 units/g of solid, Sigma) and α-amylase (porcine pancreatic, EC 3.2.1.1, 1240 units/mg of protein, Sigma). After incubation with amyloglucosidase (35 mL, 340 mg of enzyme/L of phosphate buffer, pH 4.5, 55 °C, 2 h), the samples were centrifuged (4800*g*, 4 °C, 20 min). The supernatants were removed and the samples incubated with α-amylase (35 mL, 240 mg of enzyme/L of phosphate buffer, pH 6.9, 37 °C, 2 h) and centrifuged (4800*g*, 4 °C, 20 min). The supernatant was discarded, and the pellet containing the protein was fixed in

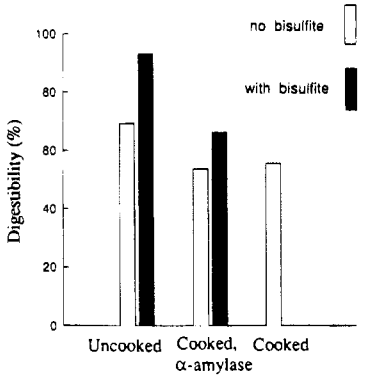

Figure 1. Protein digestibility of uncooked and cooked sorghum with and without α-amylase and the effect of addition of a reducing agent.

1% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in 0.1 M phosphate buffer, pH 6.8, for 16 h at 4 °C for examination by TEM.

Transmission Electron Microscopy. Fixed samples were rinsed in 0.1 M phosphate buffer, pH 6.8. Samples were post-fixed with 2% osmium tetraoxide in 0.05 M phosphate buffer, pH 6.8, for 4 h. Tissues were rinsed in deionized water and dehydrated in a graded ethanol series, 10 min each in 10, 30, 50, 70, 90, 95, 100, 100, and 100% ethanol. Tissues were then infiltrated for 20 min each in 20, 40, 60, and 80% LR White resin in ethanol and in 100, 100, and 100% LR White resin. Resin was replaced with fresh resin, and then samples were placed in polyurethane embedding capsules and polymerized at 55 °C in a sealed oven purged with nitrogen gas. After 48 h, samples were removed from molds and trimmed until the sample was exposed. Sections were post-stained with 2.5% (w/v) aqueous uranyl acetate for 10 min and 0.1% lead citrate for 1 min. Sections were viewed in a Phillips EM-200 (Phillips, Holland) transmission electron microscope at 60 kV.

RESULTS

Protein Extractability of Flour. The amount of protein extracted from flour under the conditions described above decreased after it was cooked (Table 1). Sonication after cooking increased extraction and digestion with α-amylase, while cooking also increased the protein extraction. When both treatments were applied, extractability increased to 101.5%. This high value may be due to residual α-amylase left in the pellet following the washings.

The extractabilities of α-, β-, and γ-kafirins from cooked flour relative to those of the uncooked flour reference values of 100.0% were 95.0, 91.9, and 88.1%, respectively. When a cooked flour mixture was sonicated, β- and γ-kafirin extraction decreased considerably. Digestion of the starch with α-amylase slightly dropped the α-kafirin extraction but increased β- and γ-kafirin extractability relative to the uncooked flour. When sonication and α-amylase treatment were used after cooking, relative extractability of α-, β-, and γ-kafirins increased slightly. We chose to use the α-amylase treatment because with only minimal manipulation of the sample we were able to significantly improved the kafirin extractability of the cooked flour without affecting protein digestibility.

Protein Digestibility. *In vitro* protein digestibility of uncooked flour was 69.2%, and it dropped to 43.6% after cooking (Figure 1). Treatment with sodium bisulfite increased protein digestibility of both the uncooked and cooked sorghum to 93.0 and 56.2%, respectively. The digestibility of sorghum flour cooked with α-amylase was approximately the same as that cooked without α-amylase.

Content of α-, β-, and γ-Kafirins in the Undigestible Residue. ELISA determination of the three kafirins (Table 2) showed that after 2 h of pepsin digestion of 200 mg of flour, the undigested residue from the uncooked sample contained 31.0% (4.52 mg) of the original α-kafirin, 15.3% (0.72 mg) of the β-kafirin, and 13.5% (0.31 mg) of the γ-kafirin. Soaking flour in sodium bisulfite greatly decreased the amount of undigested α-kafirin and reduced β- and γ-kafirins to undetectable amounts. Cooking caused the three kafirin classes to become more resistant to pepsin digestion. Indigestible protein increased in the β- and γ-kafirin classes approximately 2.5 and 2 times over the uncooked values, respectively. Addition of sodium bisulfite to the cooking water substantially increased the digestibility of α-, β-, and γ-kafirins.

Transmission Electron Microscopy. *Uncooked Sorghum Flour.* In uncooked sorghum flour, amyloglucosidase and α-amylase only partially digested the starch granules so that many granules remained (Figure 2A). Protein bodies were embedded in a dark staining protein matrix. The protein bodies had concentric rings, many dark inclusions, and dark staining projections that extended from the periphery of the protein body to the interior. After digestion with pepsin, most of the protein matrix was digested away, and the protein bodies were extensively pitted at their surfaces. Many protein bodies were almost completely digested with only a core or central fragment remaining (Figure 2B).

Cooked Sorghum Flour. Protein bodies in the undigested sample of cooked sorghum looked similar to the uncooked ones (Figure 2C). Protein bodies were embedded in a dark staining protein matrix with dark staining inclusions and projections. After pepsin digestion, however, the majority of the protein bodies were unaffected by pepsin and no pitting was observed (Figure 2D). The protein bodies in both undigested and digested samples appeared more fibrous than those in the uncooked samples.

Sodium Bisulfite-Soaked Sorghum Flour. The structure of protein bodies and matrix of sorghum flour soaked in sodium bisulfite was similar to that observed in the other undigested flour samples (Figure 3A). After incubation with pepsin, protein bodies were almost completely digested away (Figure 3B).

Sodium Bisulfite-Soaked, Cooked Sorghum Flour. Protein bodies of flour cooked in sodium bisulfite were similar to the previously described undigested samples (Figure 3C). After digestion, some protein bodies were extensively digested and many showed the pitting that was not observed in unreduced, cooked flour (Figure 3D). The partially digested protein bodies that remained appeared fibrous.

Control Samples. Samples soaked in phosphate buffer instead of pepsin solution showed no protein body digestion and were not observably different from the undigested sorghum flour samples.

DISCUSSION

As a consequence of its internal location, α-kafirin is degraded the most slowly among the kafirins in the protein bodies, even though when isolated in its unreduced form it is easily digested by pepsin (Hamaker, unpublished data, 1993). Among the kafirins, β and γ are the first ones to be digested due to their peripheral location in the protein body. Cooking may change the flour structure and promote disulfide bonding that

Table 2. Percentage[a] and Absolute Amounts of α-, β-, and γ-Kafirins in the Residue after Pepsin *in Vitro* Digestion of Flour (200 mg)

	kafirin					
	α		β		γ	
treatment	%	mg	%	mg	%	mg
uncooked	31.0 (2.3)	4.52 (1.03)	15.3 (2.8)	0.72 (0.13)	13.5 (3.0)	0.31 (0.00)
uncooked with sodium bisulfite	2.0 (0.0)	0.29 (0.00)	0.0 (0.0)	0.00 (0.00)	0.0 (0.0)	0.00 (0.00)
cooked	47.9 (6.8)	6.98 (1.03)	41.2 (3.0)	2.04 (0.13)	28.1 (2.9)	0.60 (0.05)
cooked with sodium bisulfite	12.4 (2.0)	1.81 (0.31)	6.0 (0.2)	0.31 (0.00)	12.2 (3.8)	0.23 (0.05)

[a] Expressed in percentage of the amount of α-, β-, or γ-kafirin in an uncooked, undigested control. Means of two determinations (SD).

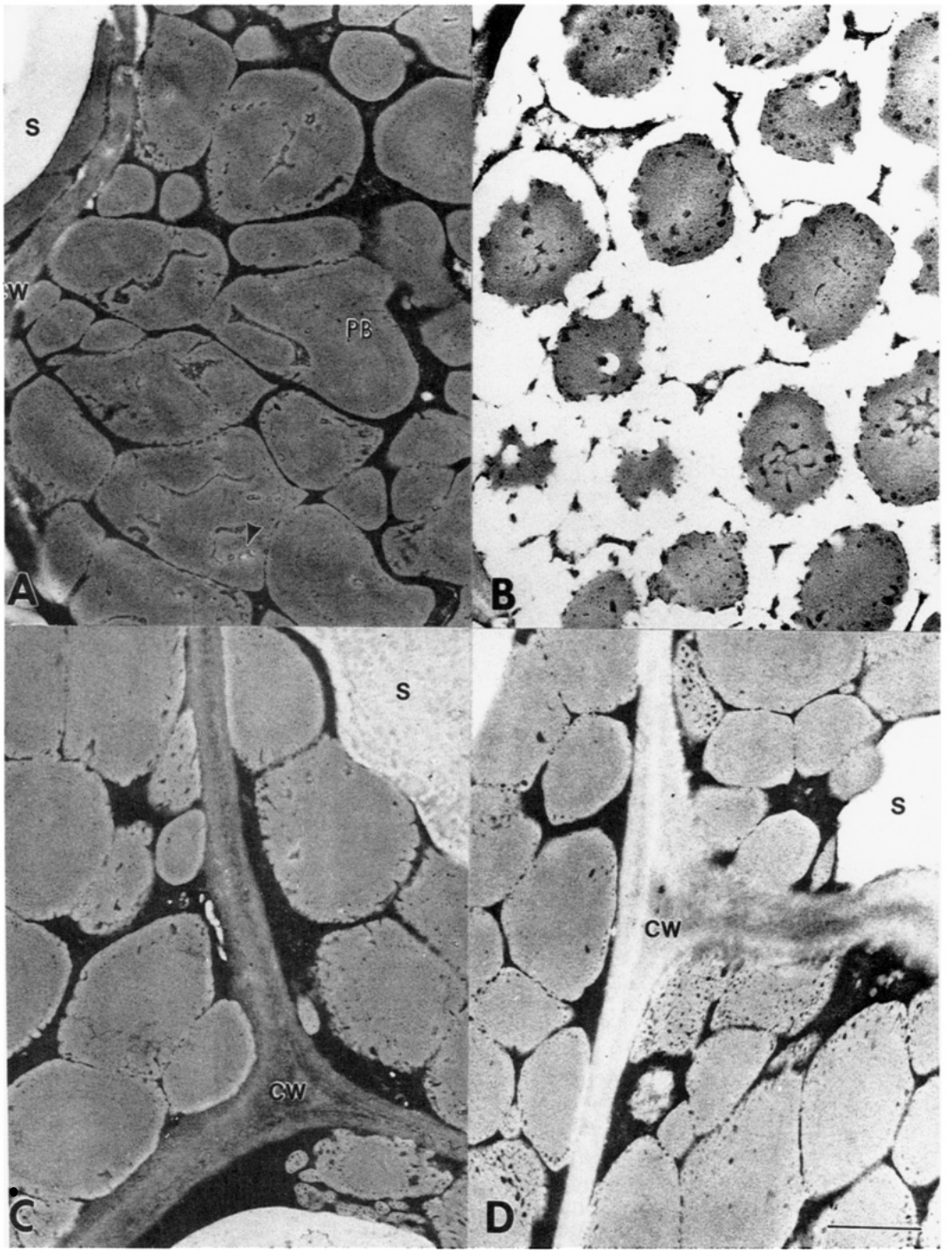

Figure 2. Electron micrographs of uncooked and cooked sorghum without sodium bisulfite treatment: (A) uncooked flour without pepsin digestion (arrow indicates protein body degradation); (B) uncooked flour after 120 min of pepsin digestion; (C) cooked flour without pepsin digestion; (D) cooked flour after 120 min of pepsin digestion. CW, cell wall; PB, protein body; S, starch. Bar = 1 μm.

negatively affects kafirin digestibility. The addition of sodium bisulfite partly reverses this effect, probably through the prevention of disulfide bond formation and cleavage of existing bonds.

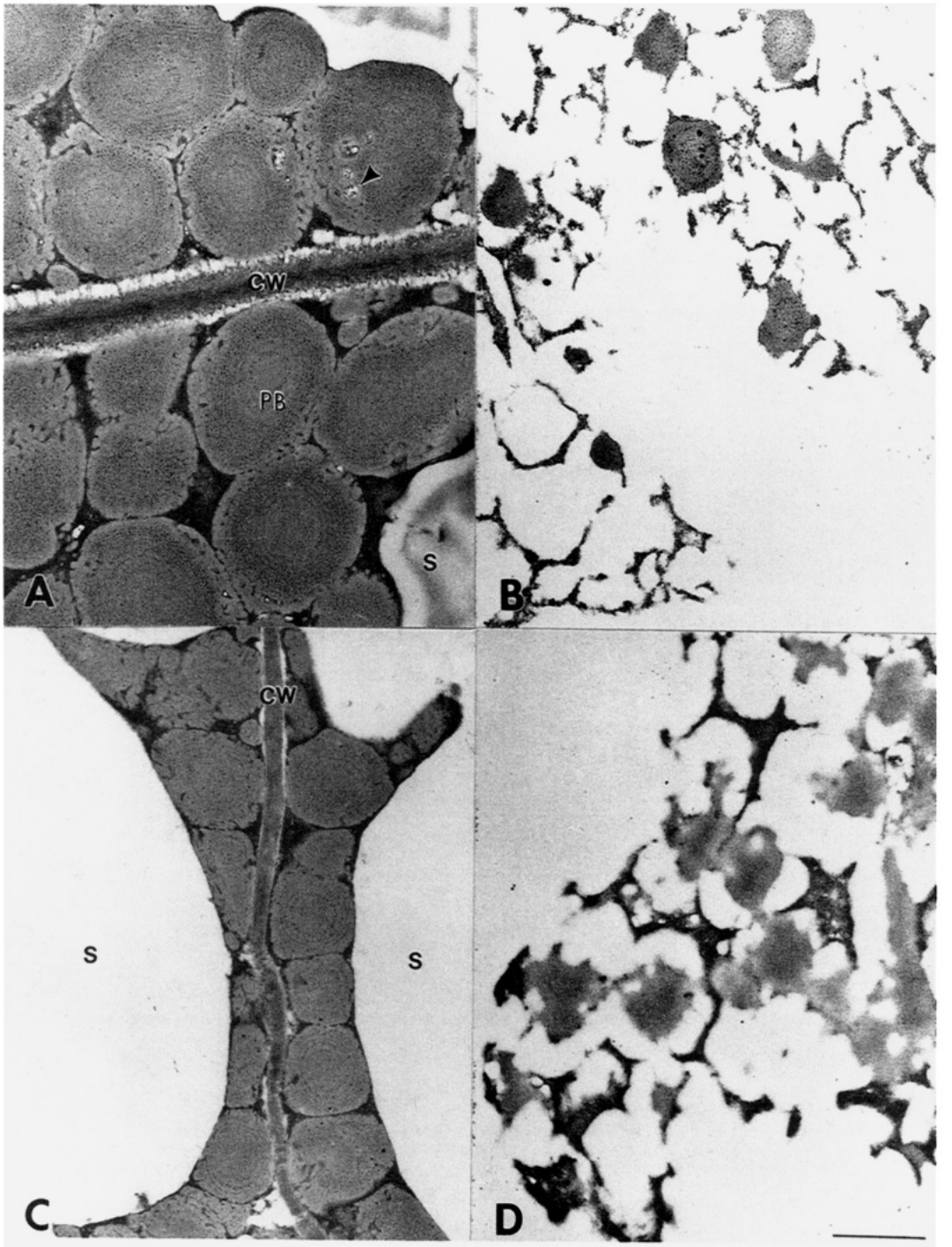

Figure 3. Electron micrographs of uncooked and cooked sorghum soaked in sodium bisulfite: (A) uncooked flour without pepsin digestion (arrow indicates protein body degradation); (B) uncooked flour after 120 min of pepsin digestion; (C) cooked flour without pepsin digestion; (D) cooked flour after 120 min of pepsin digestion.

A preliminary requirement to study proteins from uncooked and cooked sorghum was to achieve comparable protein extractability. Extraction of similar quantities of the α-, β-, and γ-kafirins from uncooked and cooked flour was sought. It was previously shown that the solubility of kafirins decreases after cooking (Hamaker et al., 1986), and our data supports that. We found that a suitable procedure to overcome this is the use of heat-stable α-amylase while cooking. When this method was used, the extractability of kafirins generally increased. Since α-amylase did not affect the protein digestibility of cooked sorghum, enzymatic treatment of flour was used for this experiment to improve kafirin extractability of the cooked samples.

The *in vitro* digestibility results are in agreement with previous findings (Hamaker et al., 1987). As expected, protein digestibility decreased after cooking and was partially reversed on addition of sodium bisulfite. According to ELISA determination, the majority of the protein remaining in the uncooked digested residue was α-kafirin (31.0% of the amount in flour). β- and γ-kafirins were more extensively digested. An experiment using ELISA to monitor protein digestion over time revealed that at all times of digestion tested, α-kafirin was less digested than the other kafirins. This experiment also showed that at 3.5 h nearly all of the protein is enzymatically cleaved to soluble peptides (data not shown). Similarly, germination studies in which diges-

tion of kafirins was observed also showed α-kafirin took longer to digest (Taylor et al., 1985; Mazhar and Chandrashekar, 1993).

These findings may appear to be contradictory to prior studies which showed that native α-kafirin (unreduced) is relatively easily digested by pepsin and that β- and γ-kafirins are more likely to form indigestible disulfide-bound complexes. Previous studies, however, have shown that α-kafirins are located predominantly in the interior of the protein bodies and that β- and γ-kafirins, in addition to being located in the interior, are also found at the surface of the protein bodies (Shull et al., 1992). TEM micrographs from our study showed that protein bodies in uncooked sorghum flour were digested by pitting from the outer surface and degradation of the protein bodies continued gradually toward the interior. Similar results were observed in the SEM study of Rom et al. (1992). This suggests that pepsin starts to act on the surface of the protein body, so it is likely that α-kafirin cannot be degraded until part of the outside of the protein body is digested away. We think that it is not the α-kafirin structure but its internal location that makes it inaccessible to enzymes and therefore less likely to be digested. Soaking the flour in a sodium bisulfite solution accelerated the digestive process so that virtually no kafirin remained, except 2% of the α-kafirin, which again confirms that it is the last one to be digested.

When the flour was cooked, the proteins that were the most affected were β- and γ-kafirins. The resistance of α-kafirin is also noted, and this again is most likely a result of its location. As viewed with TEM, many cooked sorghum protein bodies were almost unaffected by pepsin. Protein bodies did not exhibit the usual pitting observed after exposure to pepsin. This, along with the fibrous material observed on the outer edges of partially digested cooked protein bodies, suggests that a structural change occurred during cooking.

We believe enzymatically resistant protein polymers are formed during cooking of sorghum through disulfide bonding of the β- and γ-kafirins, and possibly other proteins, located at the periphery of the protein body. This would restrict digestion of the α-kafirin inside. When a reducing agent was added to the cooking water, we observed an increase in protein digestibility of all the kafirins, especially β-kafirin, when compared to the untreated cooked flour. The digestibility was not fully reversed to the level of uncooked flour, possibly due to the presence of inaccessible disulfide bonds.

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