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Improving the *in vitro* protein digestibility of sorghum with reducing agents

(pepsin digestibility/trypsin–chymotrypsin digestibility/cereals/disulfide linkages)

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ABSTRACT **We have shown in previous reports that cooked sorghum protein is less digestible than other cooked cereal proteins. The pepsin-indigestible proteins in sorghum were found to be mainly prolamin proteins. Cooking sorghum in the presence of 2-mercaptoethanol increased protein digestibility (*in vitro* with pepsin or trypsin/chymotrypsin) to a level comparable with other cereals. At a concentration of 100 mM, other reducing agents (dithiothreitol, sodium bisulfite, and L-cysteine) were equally effective in improving sorghum digestibility. When maize was cooked in the presence of 2-mercaptoethanol, protein digestibility increased 5% compared to 25% for sorghum. Cooking barley, rice, and wheat with 2-mercaptoethanol had no significant effect on protein digestibility. The addition of reducing agents appears to prevent the formation of protein polymers linked by disulfide bonds.**

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the major food crops of the semiarid regions of Africa and Asia and a major animal feed crop in the Americas and Europe. Sorghum is considered to be poor people's food and is generally consumed in rural areas, where it may comprise >70% of the food intake (1). Because sorghum is eaten in areas where the populations are frequently undernourished, it is important to consider the quality, quantity, and availability of the nutrients in the grain.

Nitrogen-balance studies conducted on children or young adults using sorghum cooked to a gruel or a bread have shown that the proteins are poorly digested relative to the proteins of other cereals (2–4). In studies with Peruvian children, MacLean *et al.* (4) reported that sorghum gruels from four tannin-free cultivars had an apparent protein digestibility mean value of 46%, while rice, maize, and wheat gruels gave values of 66%, 73%, and 81%, respectively.

Axtell *et al.* (5), using an *in vitro* pepsin digestibility method to estimate protein digestibility of the sorghums supplied to MacLean, reported that digestibility values decreased significantly after cooking, indicating that cooking may alter the sorghum proteins and make them less digestible. Cooked sorghum proteins digested with trypsin/chymotrypsin or pepsin followed by trypsin/chymotrypsin were also poorly digested compared to those of the uncooked flour (6). On the other hand, cooked maize flour was either as digestible or slightly more digestible than the uncooked flour when pepsin and/or trypsin-chymotrypsin were used.

Animal feeding studies also support the premise that cooking causes a reduction in digestibility of sorghum proteins. Mitaru *et al.* (7) found that chickens digested 31.5% less protein from cooked, whole-grain, low-tannin sorghum than from the uncooked grain. Rats fed with low-tannin sorghum that was cooked to a gruel digested 7% less protein than those fed the uncooked flour (8).

Recent findings (6) showed that cooking sorghum preferentially decreased the pepsin digestibility of the alcohol-soluble prolamin proteins, the kafirins. Cooking also substantially altered the solubility properties of the kafirins more than those of the maize prolamins (zeins).

Some methods of processing sorghum increase protein digestibility. Studies in Peru showed that apparent protein digestibility of heat-extruded decorticated sorghum was 81% in children (9), and Graham *et al.* (10) showed that a Sudanese fermented sorghum weaning product had digestibility of 79%.

Indications that sorghum proteins contain high levels of disulfide cross-linkages compared with maize and millet led Axtell *et al.*‖ to suggest that this might be responsible for the low digestibility of sorghum. It appeared logical, therefore, to test the effect of the reduction of disulfide bonds on digestibility in sorghum and other cereals.

MATERIALS AND METHODS

The cereals tested were sorghum (tannin-free P721N, 1982 crop year), maize, barley, hard red winter wheat, and rice. All grains were ground in a Udy Mill (Boulder, CO) to pass through a 0.4-mm screen. *In vitro* protein digestibility tests were modified from the method of Mertz *et al.* (11). Enzymes used were pepsin (Sigma P-7000; activity 1200 units per mg of protein) and a trypsin/chymotrypsin mixture {Sigma T-8128; trypsin activity, 1330 BAEE units [one BAEE unit = ΔA_{253} of 0.001 per min with $N\alpha$-benzoyl-L-arginine ethyl ester (BAEE) as substrate in 3.2 ml at pH 7.6 at 25°C]; chymotrypsin activity, 1260 ATEE units [one ATEE unit = ΔA_{237} of 0.001 per min with *N*-acetyl-L-tyrosine ethyl ester (ATEE) in 3 ml at pH 7.0 at 25°C]}. Uncooked flour (200 mg) was either used directly or soaked for 12 hr at 4°C in 2 ml of water with or without different concentrations of 2-mercaptoethanol. Cooked materials were prepared by suspending 200 mg of flour in 2 ml of water or 2 ml of water containing 100 mM 2-mercaptoethanol, dithiothreitol, sodium bisulfite, or L-cysteine and stirred in a boiling water bath for 20 min. Samples were then suspended in 35 ml of a buffer containing pepsin or trypsin/chymotrypsin (1.5 g of enzyme per liter of 0.1 M KH_2PO_4 buffer, pH 2.0 and 7.6, respectively) and incubated in a shaking water bath at 37°C. Pepsin digestion was stopped at 2 hr with the addition of 2 ml of 2 M NaOH. Trypsin/chymotrypsin digestion was stopped by placing the digestion flasks on ice. After centrifugation at 4800 × *g* at 4°C for 20 min, the supernatant was discarded, and the residue was washed in 15 ml of buffer and recentrifuged. The residue was analyzed for nitrogen by micro-Kjeldahl digestion and colorimetric nitrogen analysis (12). Indigestible nitrogen was

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‖Axtell, J. D., Ejeta, G. & Munck, L., Sorghum in the Eighties: Proceedings of the International Symposium on Sorghum (ICRISAT), Nov. 2–7, 1981, Patancheru, A. P., India, pp. 589–603.

Table 1. Pepsin digestibility of uncooked and cooked cereals

Cereal	% digestibility*		
	Uncooked	Cooked	Decrease
Sorghum	80.8	56.3	24.5
Maize	83.4	79.3	4.1
Barley	93.2	80.2	13.0
Rice	91.1	82.1	9.1
Wheat	91.3	85.9	5.4

*Triplicate determinations.

subtracted from total nitrogen, and the percentage of soluble nitrogen was reported as *in vitro* protein digestibility.

RESULTS

Of the five cereals tested, sorghum had the lowest protein digestibility, whether it was digested with pepsin in the uncooked or cooked form (Table 1). In the uncooked group, sorghum and maize were about 10% less digestible than barley, rice, or wheat. When cooked, however, only sorghum exhibited the large decrease in digestibility (24.5%). The fact that cooked sorghum protein digestibility was significantly lower than that of the other cereals is in agreement with findings of MacLean *et al.* (4) and Mertz *et al.* (11). The other cereals, especially barley and rice, also showed some decrease in pepsin digestibility after cooking, though not nearly to the degree of sorghum. In a previous study (6), we did not observe a decrease in the digestibility of maize after cooking; we attribute the decrease in the current study (4%) to the modification of the pepsin assay—namely, stopping the enzymatic reaction with alkali.

Treating sorghum with 2-mercaptoethanol resulted in large increases in *in vitro* protein digestibility (Table 2). The reducing agent affected both uncooked and cooked sorghum and resulted in increasing pepsin digestibility by 11.1% and 25.1%, respectively, when compared with sorghum that was soaked or cooked in water alone. Preliminary work showed that pepsin digestibility was maximally increased by treating samples with 10 mM 2-mercaptoethanol solution for uncooked sorghum and 100 mM 2-mercaptoethanol for cooked sorghum (Fig. 1). Therefore, 100 mM 2-mercaptoethanol was used to treat both uncooked and cooked samples. Addition of higher concentrations of 2-mercaptoethanol (up to 200 mM) showed no improvement nor inhibition in protein digestibility. Digestion with a mixture of trypsin and chymotrypsin in the presence of 2-mercaptoethanol also resulted in much higher digestibilities than for the untreated material (Table 2). Trypsin/chymotrypsin digestibilities of the reduced preparations increased by 10.6% and 23.5% for the uncooked and cooked sorghum, respectively.

Other reducing agents also enhanced protein digestibility of sorghum (Fig. 2). When sorghum was cooked in 100 mM solutions of dithiothreitol, sodium bisulfite, or L-cysteine, pepsin digestibilities increased by 27.3%, 25.0%, and 20.3%,

Table 2. *In vitro* digestibility of sorghum soaked or cooked in the presence of 2-mercaptoethanol

Digest	% digestibility*			
	Uncooked†		Cooked‡	
	NT	HSC_2H_4OH	NT	HSC_2H_4OH
Pepsin	83.2	94.3	56.7	81.8
Trypsin/chymotrypsin	62.4	73.0	59.4	82.9

*Triplicate determinations.
†Uncooked flours were soaked either in water (NT) or a solution containing 100 mM 2-mercaptoethanol for 12 hr prior to digestion.
‡Cooked gruels were prepared either in water (NT) or in a solution containing 100 mM 2-mercaptoethanol.

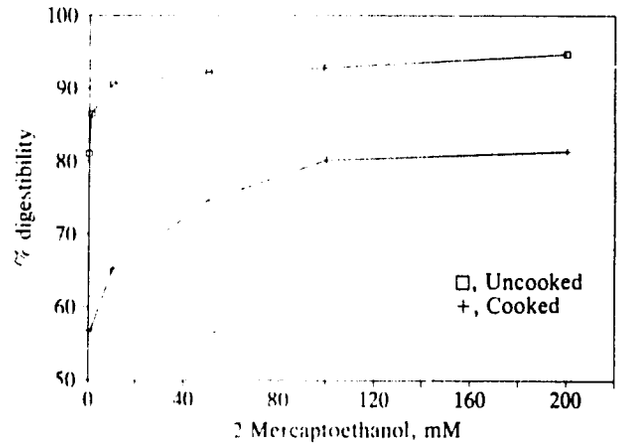

FIG. 1. Pepsin digestibility of sorghum with addition of different amounts of 2-mercaptoethanol

respectively, over sorghum cooked in water alone. Dithiothreitol was most effective, followed by bisulfite, 2-mercaptoethanol, and L-cysteine, in increasing pepsin digestibility at low concentrations. At 100 mM, however, the effect of the reducing agents on digestibility was nearly equal.

The addition of 2-mercaptoethanol increased the pepsin digestibility of sorghum much more than it did the digestibility of the other cereals tested (Table 3). After the cereals were treated with 2-mercaptoethanol and then soaked or cooked, sorghum digestibility was comparable to that of the other cereals. Addition of a reducing agent also increased the digestibility of uncooked and cooked maize proteins, but to a lesser extent than in sorghum. Other cereals (barley, rice, or wheat) were not appreciably affected by the presence of 2-mercaptoethanol

DISCUSSION

Sorghum proteins behaved differently from the proteins of other cereals. Their digestibility was most negatively affected by the cooking process and most enhanced by the addition of reducing agents. Since the presence of a reducing agent allows cooked sorghum proteins to be more easily digested, rearrangement or oxidative formation of disulfide bonds during the cooking process presumably plays a part in creating the less digestible proteins in sorghum gruel. As it has already been established that the kafirin proteins become less digestible after cooking (6, 13), it appears that the formation of disulfide linkages preferentially affects the digestibility of the kafirins.

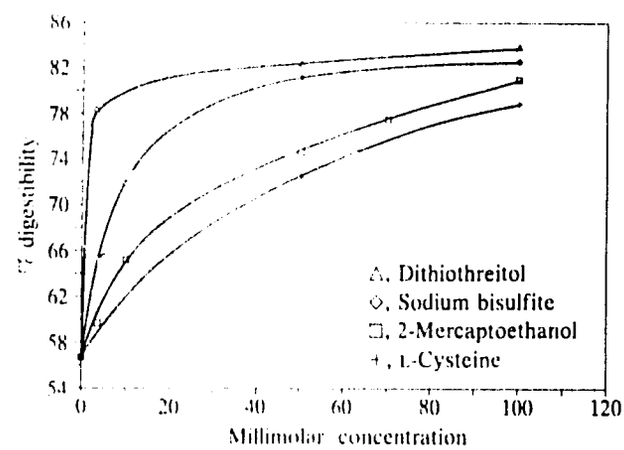

FIG. 2. Effect of different reducing agents on the pepsin digestibility of cooked sorghum

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Table 3. Effect of 2-mercaptoethanol on the pepsin digestibility of major cereals

	% digestibility*					
	Soaked†			Cooked‡		
Cereal	NT	HSC_2H_4OH	Increase	NT	HSC_2H_4OH	Increase
Sorghum	83.2	94.3	11.1	56.7	81.8	25.1
Maize	86.4	94.8	8.4	78.0	83.1	5.1
Barley	90.2	90.7	0.5	79.6	80.9	1.3
Rice	91.1	94.6	3.5	80.8	81.8	1.0
Wheat	94.5	94.5	0.0	86.3	86.6	0.3

*Duplicate determinations.
†Uncooked flours were soaked either in water (NT) or a solution containing 100 mM 2-mercaptoethanol for 12 hr prior to digestion.
‡Cooked gruels were prepared either in water (NT) or a solution containing 100 mM 2-mercaptoethanol.

We wish to propose two theories to explain why the kafirins are less digestible after cooking. First, kafirin proteins may form polymeric units bound by intermolecular disulfide bonds. The polymers may be less susceptible to digestion compared to the lower molecular weight protein units that are presumably present in the flour. This seems to be a reasonable hypothesis, since kafirin proteins, even in their monomeric state, may be more resistant to enzymatic digestion than the other sorghum proteins because of their highly hydrophobic nature. Polymers of kafirin would likely be even more difficult to digest. Although kafirin proteins have not been analyzed as such, maize zeins have been shown to be more resistant to digestion than casein (14). Other workers (15, 16) have also implied that the more insoluble a protein is, the less susceptible it is to digestion.

Second, lowered digestibility simply may be caused by the inaccessability of the protein bodies to enzymatic attack. Protein bodies are well-defined structures in the sorghum endosperm and contain the kafirin proteins. By using scanning electron microscopy, it was found that protein bodies remain intact after cooking and are even present after pepsin digestion (Arun Chandrashekar and A.W.K., unpublished data). It is possible, then, that cooking makes at least some of the protein bodies more resistant to digestion. This could be due to formation of either a disulfide-bound protein coat produced by proteins surrounding the protein body or an interior "toughening" of the periphery of the protein body because of disulfide bond formation.

Other researchers have shown that heat treatment can enhance the formation of disulfide linkages, which in turn produce less digestible proteins. Opstvedt *et al.* (17) showed fish muscle to be 3% less digestible after heat treatment, which was correlated to an increase in disulfide bond formation. Belikov *et al.* (18) heated proteins containing high amounts of cysteine and found them to be less digestible than the unheated proteins, while the digestibility of heated low-cysteine proteins was not changed. This indicated that disulfide bonds were being formed during heating of the high cysteine/cystine proteins.

Maize was the only other cereal in which protein digestibility was substantially improved by the addition of 2-mercaptoethanol. The maize prolamins (zeins) also have been shown to be the major pepsin- and trypsin/chymotrypsin-indigestible protein of uncooked and cooked maize flour (E.R.H. and A.W.K., unpublished data). This may be explained by the fact that zeins, like kafirins of sorghum, are found in protein bodies. Unlike wheat, the protein bodies from sorghum and maize remain intact in the mature grain and may form a barrier preventing efficient digestion of the prolamins. The *in vitro* protein digestibility of cooked maize was slightly lower than that of the flour (Table 1) and was not affected by 2-mercaptoethanol to the degree observed with sorghum (Table 3). However, the digestibility of the maize flour increased 8.4% when treated with 2-mercaptoethanol, which was about the same as the improvement found for treated sorghum flour (Table 3).

The reason for the digestibility increases of both uncooked sorghum and maize flour when 2-mercaptoethanol was added may be fairly simple. The protein bodies of both are located between the starch granules, and many are probably embedded in the protein matrix. This matrix is made up primarily of glutelin proteins that are thought to exist in the form of polymers bound by intermolecular disulfide linkages (19). Thus, reducing agents could conceivably open up the protein matrix through the cleavage of disulfide bonds and, thereby, allow the digestive enzymes more accessibility to the protein bodies.

Sorghum is used as the staple food crop by millions of people (1), many of whom have nutritionally inadequate diets. Two processes, fermentation (10) and heat extrusion (9), have already been shown to increase the digestibility of sorghum in children. The fact that the decrease in digestibility of sorghum normally seen following cooking of the flour to a gruel can be reversed by the addition of disulfide-cleaving agents should make it possible to determine or identify other methods of food preparation that would lead to highly digestible sorghum foods. Since malnutrition is not uncommon in the sorghum-consuming regions of the world, there is potential benefit from these findings.

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