

Proteolytic Activity in Sorghum Flour and Its Interference in Protein Analysis¹

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Cereal Chem. 77(3):343-344

An alkaline solvent containing SDS and reducing agent is commonly used for extraction of sorghum and maize proteins before further analysis (SDS-PAGE or enzyme-linked immunosorbent assay [ELISA]) (Wallace et al 1990; Hamaker et al 1995; Oria et al 1995). It is also used as the final extraction solvent in the Landry-Moureaux (1970) protein fractionation method. The solvent composition used by Wallace et al (1990) for extraction of essentially the total grain protein contains 1% SDS and 2% 2-mercaptoethanol (2-ME) in a borate buffer at pH 10. During routine analysis of extracted sorghum proteins using SDS-PAGE, we noticed that, for a few sorghum cultivars, protein bands appeared hydrolyzed to a significant extent. Bands representing both kafirin (the sorghum storage protein), and nonkafirin proteins were nearly eliminated from the gel, and there was an accumulation of low molecular weight peptides. As this suggested the presence of a protease active under the extraction conditions, we further investigated this possibility.

In this brief report, a solvent-active protease is revealed to be present to some degree in most stored sorghum flours. Its origin appears to be fungal. The significance of this finding rests in the interference that this protease causes in sorghum, as well as millet, grain protein analyses.

MATERIALS AND METHODS

Sample Preparation

Sorghum cultivars (P721Q, SC283-14, Sepon 82, SRN39, P721N, crop year 1993; and SRN39, crop year 1996) were grown and stored at the Purdue Agronomy Farm, West Lafayette, IN. Other cereals including wheat (BPS4), maize (W64A+), rice (IR29), and pearl millet (population MLS, University of Nebraska) also were used. Whole grain samples were ground through a 0.5-mm mesh screen to flour using a cyclone mill (Tecator Cyclotec, model 1093, Sweden).

In addition, sorghum grain cultivars P721Q, SC283-14, Sepon 82, P721N and SRN39 (1993) were decorticated using a tangential abrasive dehulling device (model 4E-110, Venable Machineworks, Canada) and then hand-degermed using an adjustable speed drill (Dremel, Racine, WI) to remove any remaining germ. The endosperm was ground to flour in a ball mill for 4 min at the highest speed setting (Retsch, type MM-2, Germany).

Protein Extraction and SDS-PAGE

Whole grain flour and decorticated and hand-degermed flour (50 mg) were incubated for 4 hr at room temperature ($\approx 22^{\circ}\text{C}$) in extraction solvent (0.0125M sodium borate, 1% [w/v] SDS, 2% [v/v] 2-ME, pH 10.0) at a flour-to-solvent ratio of 1:15 (w/v). The suspension was spun in a microcentrifuge at $20,000 \times g$ for 10 min. Supernatant (200 μL) was mixed with sample solvent (2% [w/v] SDS, 2% [v/v] 2-ME, 0.066M Tris, pH 6.8 with 10% [v/v] glycerol and 0.01% [w/v] bromophenol blue) in a ratio of 2:1 (v/v), and heated in a boiling water bath for 3 min.

The SDS-PAGE procedure was performed on a minigel-electrophoresis system (Protean II, Bio-Rad, Richmond, CA). The separating gel was a 10–15% (w/v) polyacrylamide linear gradient with a 4% (w/v) stacking gel. Prepared samples (20 μL) and a broad range molecular weight standard (6,500–200,000) (Bio-Rad) were applied to the wells of the gel. Electrophoresis was performed in the tank buffer (0.025M Tris, 0.19M glycine, 1% SDS, pH 6) at 200V for 45 min. Proteins were stained with Gelcode blue stain reagent (Pierce, Rockford, IL) and destained with water.

A time-course extraction at 1, 2, 5, 10, and 15 hr on whole grain flour of SRN39 (crop year 1996, chosen because of its high proteolytic activity) was performed, using a 2-hr extracted sample treated with 20 mM phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, as the control. In preliminary experiments, a variety of protease inhibitors (Bestatin, EDTA, E-64, and PMSF) were tested and PMSF was found to be most effective in eliminating proteolysis (data not shown). An examination of solvent-active protease activity in sorghum (SRN39), wheat (BPS4), maize (W64A+), rice (IR29), and pearl millet (population MLS, University of Nebraska) also was conducted, with PMSF-treated samples as controls.

Reduction in intensity of the SDS-PAGE α -kafirin band during the time-course incubation was measured using a digital camera and image analysis software (Kodak Digital Science Electrophoresis Documentation and Analysis System).

Gelatin-Impregnated SDS-PAGE

To identify the protease and to estimate its molecular weight (determined using a calibration plot of the log of marker molecular weight vs. the distance of each band from the top of the gel), a 0.15% (w/v) gelatin-impregnated 8% (w/v) SDS-PAGE gel was used, with clear zones indicating proteolytic activity. The gel was preelectrophoresed for 30 min at 200V in the system used above to remove excess gelatin. After sample application it was run for ≈ 25 min. Clean, sound grain and weathered grain (fungal infection identified as discolored, damaged, or with black mold on the grain) of cultivar SRN39 (1996) were separated by hand and ground to flour, and 70 mg was extracted in 300 μL of the solvent for 3 hr. The suspension was centrifuged and supernatants (15 μL) were applied to the wells of the gel. The protease was identified as a clear band at the base of the clear zone that began at the top of the separating gel. The zone represented proteolysis of the gelatin that occurred during electrophoresis as the protease migrated through the gel.

RESULTS AND DISCUSSION

After a relatively long incubation (4 hr) of whole grain sorghum flour in extraction solvent, varying degrees of proteolysis were observed in proteins of all sorghum cultivars tested (P721N, P721Q, SC283-14, Sepon 82, and SRN39) (Fig. 1a). Decorticated and degermed grains showed no proteolysis, indicating that the protease of question resides in the nonendosperm regions of the grain. Cultivar SC283-14 had the least amount of proteolysis and SRN39 the highest, resulting in completely degraded proteins. PMSF-treated flour of sorghum cultivar SRN39 showed no proteolysis in a 2-hr extraction compared with a high degree of proteolysis in the untreated sample (Fig. 1b), thus verifying that the responsible agent was a solvent-active protease. A time-course incubation of SRN39 showed rapid degradation of the proteins (Fig. 1b). Image analyses of the predominating α -kafirin storage

¹ Paper No. 15966 from the Purdue Agricultural Experimental Station

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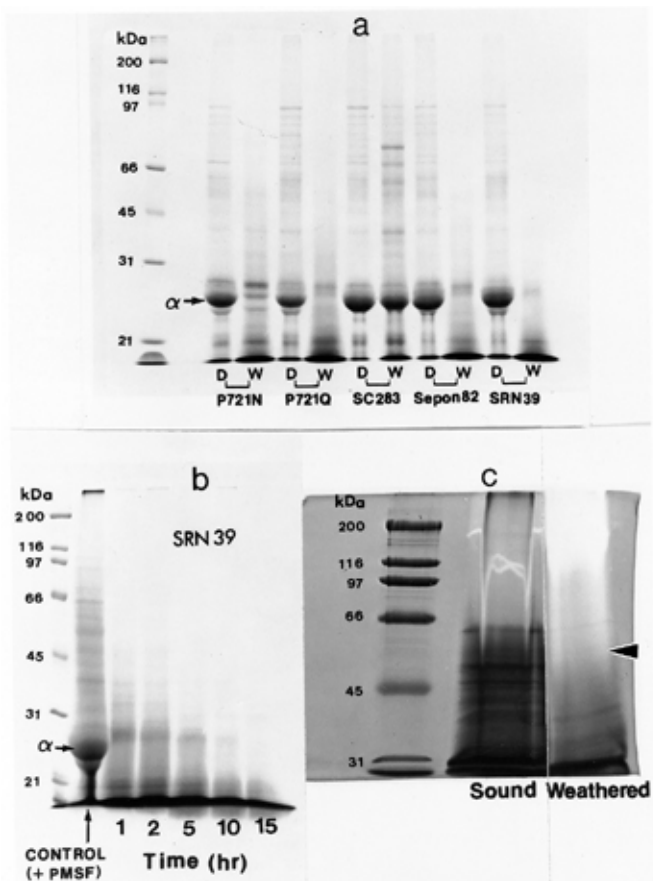


Fig. 1. (a) SDS-PAGE (10–15% gradient gel) of protein extracted from decorticated and degermed (D), and whole grain (W) flours from sorghum cultivars P721N, P721Q, SC283-14, Sepon82, and SRN39. (b) SDS-PAGE (10–15% gradient gel) time-course protein extractions of flour from cultivar SRN39. (c) Gelatin-impregnated SDS-PAGE (8% gel) of protein extracts of flour from sound and weathered SRN39 grains. α = α -kafirin band.

protein band intensity showed a \approx 90% reduction in band concentration after 1 hr of extraction compared with the PMSF control. At 10 hr of extraction, the protein was nearly completely degraded.

Figure 1c shows a gelatin-impregnated SDS-PAGE gel of sorghum protein extracts of flour from grain visually separated on the basis of whether it appeared sound or weathered (fungal infected). Much higher protease activity was observed in flour made from weathered grain than sound grain, as evidenced by the degradation of proteins and the presence of the clear gelatin-digested zone in the gel (Fig. 1c, last lane). It was notable and surprising that the protease was active even under electrophoresis conditions. The absence of pronounced clear zone in the SDS-PAGE profile from sound grain suggested that there was very little protease in the absence of visual weathering. Therefore, these data strongly suggest that the solvent-active protease is of fungal origin. SDS-PAGE analysis of flour of the five sorghum cultivars (extracted for 4 hr) that were freshly harvested from the field showed no hydrolysis (not shown). It is possible, then, that fungal growth and protease production may occur during storage, however, this aspect needs to be studied further. Further studies also need to be done to confirm that the protease is fungal produced and not an intrinsic protease.

An estimate of the molecular weight of the solvent-active protease was determined from the faint clear band appearing at the base of the clear zone (Fig. 1c, last lane, arrow). The protease had an M_r of \approx 60 kDa. When the gel was electrophoresed under cold conditions and afterward allowed to incubate, the clear band representing the protease was more pronounced (not shown). Comparison of different cereal grains (Fig. 2) extracted with and without the protease

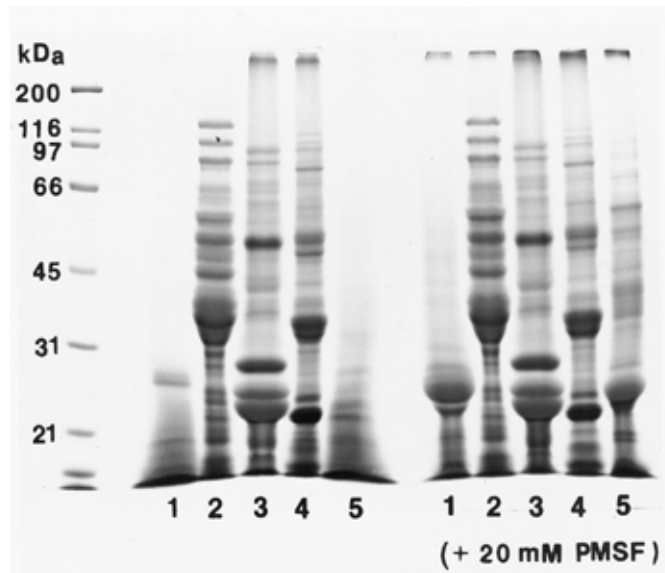


Fig. 2. SDS-PAGE (10–15% gradient gel) of protein extracted from whole grain flour from different cereals. Lanes: 1, sorghum; 2, wheat; 3, maize; 4, rice; 5, pearl millet.

inhibitor PMSF showed that, in addition to sorghum, pearl millet also exhibited proteolysis. The wheat, maize, and rice flour samples tested showed no solvent-active protease activity. Addition of 20 mM PMSF effectively stopped proteolysis in sorghum and millet. While this is not conclusive evidence that only sorghum and millet could contain the protease, both of these cereals are related and produce grain on exposed panicles, suggesting that similarities cause the protease to be present. Moreover, weathering of sorghum and millet grains is a significant problem in regions where rainfall occurs after grain maturation.

CONCLUSIONS

Proteolytic activity was found in whole grain flours of sorghum and pearl millet grains as evidenced from degraded proteins in SDS-PAGE banding profiles. The protease was present in the bran and germ portion of the grain. It was highly stable in a denaturing solvent and was even active under electrophoresis conditions. In other studies from our laboratory (not shown), it was found that there was also interference due to the protease in ELISA quantitation of sorghum kafirins. Similar to the above described SDS-PAGE experiments, suspected low kafirin values from ELISA were notably increased when PMSF was added to the extraction solvent. Hydrolysis of the kafirins apparently removed some epitopes. Therefore, for analytical studies on sorghum or pearl millet proteins involving similar solvent extractions, it is important to either remove the bran portion by decortication or to use a protease inhibitor to prevent proteolysis of grain proteins.

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[Received April 26, 1999. Accepted January 19, 2000.]