Sequential in Vitro Pepsin Digestion of Uncooked and Cooked Sorghum and Maize Samples

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An in vitro protein digestion study, using pepsin, was carried out in uncooked and cooked sorghum and maize flour samples. The digestibility values from the uncooked samples showed that sorghum presents digestibility values similar to those of maize. In the case of the cooked samples, it was found that a wet cooking procedure promotes a decrease in sorghum protein digestibility when compared to maize. Electrophoresis was used to follow the in vitro pepsin sequential digestion procedure, and infrared spectroscopy was applied to establish its efficiency. SDS-PAGE results showed that both uncooked samples (sorghum and maize) behave in a similar way. The wet cooking procedure increases the amount of high molecular weight aggregates and promotes the appearance of two nonreducible and nondigestible 45 and 47 kDa proteins. These two protein fractions are directly related to the loss of digestibility. It was also shown that in cooked sorghum the monomers (γ-, α-, and β-) are more resistant to digestion than the corresponding uncooked samples.

INTRODUCTION

Sorghum bicolor (L.) Moench is a cereal resistant to water drought and extreme high temperatures. Sorghum is a staple food in many areas of Africa, India, Middle East Pakistan, and northern China. As in those areas the populations are frequently undernourished, it is important to consider sorghum protein digestibility. Several studies have shown that protein digestibility of sorghum is lower than the protein digestibility of other cereals. This difference increases when the sorghum flour is submitted to a wet cooking procedure (*1*, *2*).

Concerning sorghum and maize proteins, prolamins (known, respectively, as kafirins and zeins) are the most abundant, making up 70−80% of the total endosperm protein. These proteins are located in protein bodies and have no other known function apart from storage (*3*).

A nomenclature based on the existent nomenclature for zeins was proposed for kafirins. Therefore, polypeptides with M_r of 23 and 25 kDa are named α-kafirins, and those with M_r 20 and 28 kDa are referred to as β-kafirin and γ-kafirin, respectively (*4*).

Several in vitro protein digestibility methods have been applied to study zein and kafirin digestibility. These methods are based on common basic steps and differ in minor conditions such as time of digestibility, extraction time, and/or type of enzyme used (*5−14*).

In the work presented here, protein digestibility was determined in one maize variety and in four sorghum varieties. To establish the protein digestibility a sequential in vitro protein digestibility was performed in all cereal samples. To investigate the efficiency of the extraction method used, a spectroscopic technique was applied.

MATERIALS AND METHODS

Biological Material. Cereal samples (African cultivars) consisted of one maize (PAN 6043) and four sorghum varieties (NK 283, KLW, KAT 369, and PAN 8564). Whole grain samples were ground with a coffee mill to pass through a 0.4 mm screen. The cooked samples were obtained by mixing 1 part of ground flour with 5 parts of water. The mixture was placed in a boiling water bath for 20 min. After cooking, the samples were freeze-dried and ground again.

Sequential in Vitro Protein Digestibility Procedure. The method used was adapted from previous studies (*5−14*). Pepsin (Sigma, P-7000, 975 units/mg of protein) was used for the in vitro protein digestibility study. For each variety, 11 × 60 mg flour samples were stirred and digested with pepsin (20 mg of pepsin/mL of 0.1 M KH$_2$PO$_4$, pH 2, buffer) in a water bath (37 °C) for 0, 1, 3, 5, 10, 20, 30, 60, 90, and 120 min. After these periods of time, the digestions were stopped by the addition of 100 μL of 2 M NaOH, and each tube was placed in an ice bath. Simultaneously, control samples were carried out under the same conditions, using 1 mL of 0.1 M KH$_2$PO$_4$, pH 2, buffer instead of pepsin; and after 120 min, 100 μL of 2 M NaOH was added.

All samples were centrifuged (4000 rpm at room temperature) for 3 min and the supernatants discarded. The residues were washed with 1 mL of 0.1 M K$_2$HPO$_4$, pH 7, buffer, centrifuged, and washed again with 1 mL of water. After this procedure, a set of undigested protein residues was obtained (UPR). The same procedure was performed for the cooked samples.

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Protein Determination. After the sequential digestion, all residues were freeze-dried. The 0 and 120 min digestion residues were submitted to determination of total N by elementary analysis.

Undigested Protein Extraction. Fifty milligrams of residues (UPR) of uncooked and cooked samples was submitted to protein extraction with 0.5 mL of 0.0125 M $Na_2B_4O_7 \cdot 10H_2O$ (pH 10), 2% (m/v) SDS, and 1% (v/v) 2-mercaptoethanol (*15*) to analyze the nondigested proteins. After 1 h of extraction, the mixtures were centrifuged (4000 rpm, room temperature) for 3 min. Supernatants, with the pepsin nondigested proteins (undigested protein extract, UPE), were obtained. The corresponding residues were washed with 2 mL of water to obtain the starch residue (SR) and freeze-dried.

UPE—SDS-PAGE Study. UPE samples were prepared for SDS-PAGE by mixing 10 μL of the protein extract with an equal volume of SDS-PAGE sample buffer [2% (w/v) SDS, 0.0625 M Tris, 10% (w/v) glycerol, 0.01% bromophenol blue, pH 6.8]. The samples were heated, for 5 min, in a boiling water bath; 7 μL was applied in a 15% acrylamide SDS-PAGE gel (Laemmli method) and the gel run in a Mini-Protean II electrophoretic cell with Power Pac 300 (Bio-Rad). Electrophoresis was conducted at 150 V for 1.5 h until the tracking dye, bromophenol blue, reached the bottom of the resolving gel. Gels were stained with Coomassie Blue R (Pharmacia) and destained with 40% methanol and 10% acetic acid (*16*).

Analysis of SDS-PAGE Images. Electrophoretic gels were digitalized without previous drying, in a Hewlett-Packard ScanJet 3600C scanner. The acquired image was converted into a matrix representative of the different color intensities (color-coded images) (*17*).

Each of the electrophoretic lanes was split out and separately submitted to a mathematical treatment based on joint density probability estimation (*18*). As a result, two types of matrices can be recovered, defined as difference and independence. The independence matrix is calculated from the product of the bottom and right margins of the original data matrix. The bottom margin is proportional to the mean of each column, whereas the right margins are proportional to the mean of each row. Therefore, this matrix is the product of two sets of average. This matrix is smoother than the original one; that is, it corresponds to a noiseless color-coded image. Let us envision a matrix $\mathbf{X}_{(n,m)}$, where n is the number of rows and m the number of columns. The right and bottom margins (r, b) are defined, respectively, by

$$r_i = \sum_{j=1}^{m} x_{ij} \quad \text{and} \quad b_j = \sum_{i=1}^{n} x_{ij}$$

where i is the row index and j the column index.

The independence matrix, $\mathbf{Q}_{(n,m)}$, is then defined by the product of $\mathbf{r}_{(n,1)}$ and $\mathbf{b}_{(m,1)}$ margins vectors:

$$\mathbf{Q}_{(n,m)} = \mathbf{r}_{(n,1)} \mathbf{b}^T_{(1,m)}$$

The concept behind the independence matrix is that if the rows (where the molecules are to be found) and columns (related to the migration of those molecules) of the original matrix are independent, the independence matrix is equal to the original matrix. However, the experimental data are subject to physicochemical phenomena that introduce changes in the shape of the bands (e.g., friction and changes in density); therefore, by calculating the independence matrix, these effect are eliminated to a certain extent. On the other hand, the difference matrix accounts for all of the variability not modeled by the independence matrix (e.g., friction and changes in density). Each band of the independence matrix represents, thus, a profile that is smoother than the original band and where its maximum is recovered to perform quantitative analysis.

This mathematical treatment acts as a filter that enhances protein concentration determination. All electrophoretic profile regions corresponding to electrophoretic spots were submitted to area estimation using the program Origin from Microcal Software Inc.

SR—FT-IR Study. SR samples from all uncooked and cooked samples were analyzed by FT-IR. The FT-IR spectra were obtained
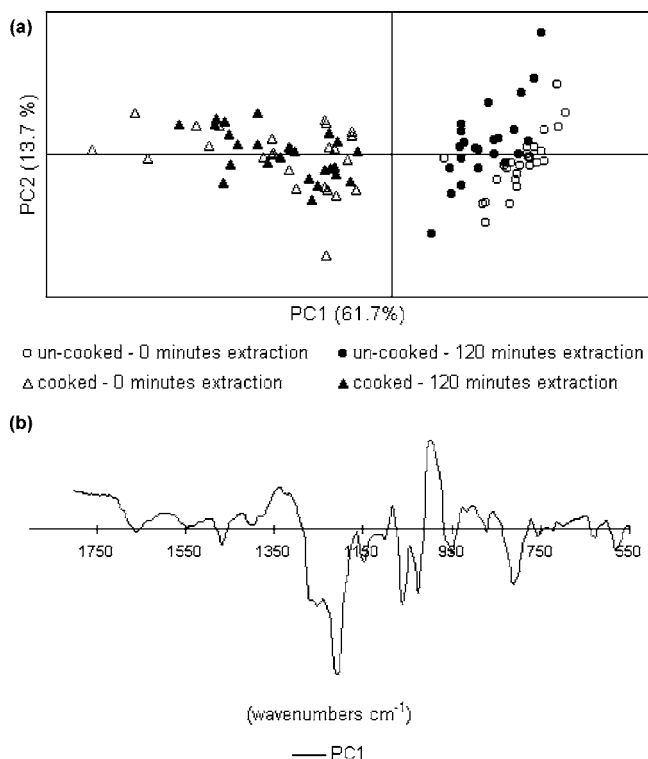
Figure 1. (**a**) PCA scores scatter plot (PC1 vs PC2) of the FTIR spectra and (**b**) PCA loadings plot profile (PC1) of the FTIR spectra; uncooked and cooked for 0 and 120 min pepsin digestion SR samples (all flour varieties), in the 1780–800 cm^{-1} region.
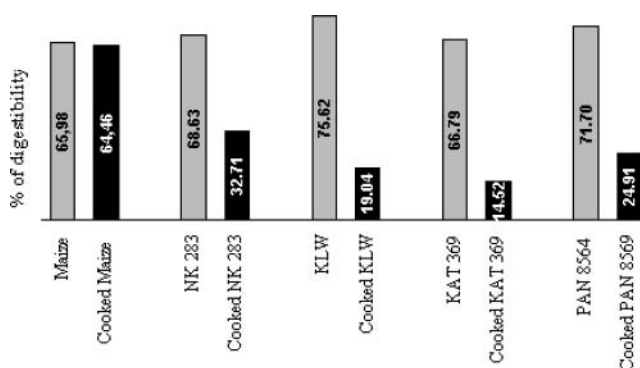

Figure 2. Percentage of digestibility, determined in UPR samples using the nitrogen content at 0 and 120 min, of uncooked and cooked maize and sorghum flours.

using a Golden Gate single-reflection diamond ATR system in a Bruker IFS-55 spectrometer. The spectra were recorded in absorbance mode from 4000 to 500 cm^{-1}, co-adding 256 scans at 8 cm^{-1} resolution. Five replicates were collected for each sample. The obtained spectra were transferred into the CATS data analysis package (*19*). For PCA analysis, the 1780−800 cm^{-1} region was autoscaled (centered and divided by the standard deviation). Each spectrum was SNV corrected (standard normal variate). The PCA analysis allowed the characterization of the sample relationships (scores plans) and the recovery of their subspectral profiles (loadings) (*20*).

RESULTS AND DISCUSSION

SR—FT-IR Study. The efficiency of the extraction of the proteins is an issue that has not been discussed before. For the correct interpretation of the digestion results, it is important to
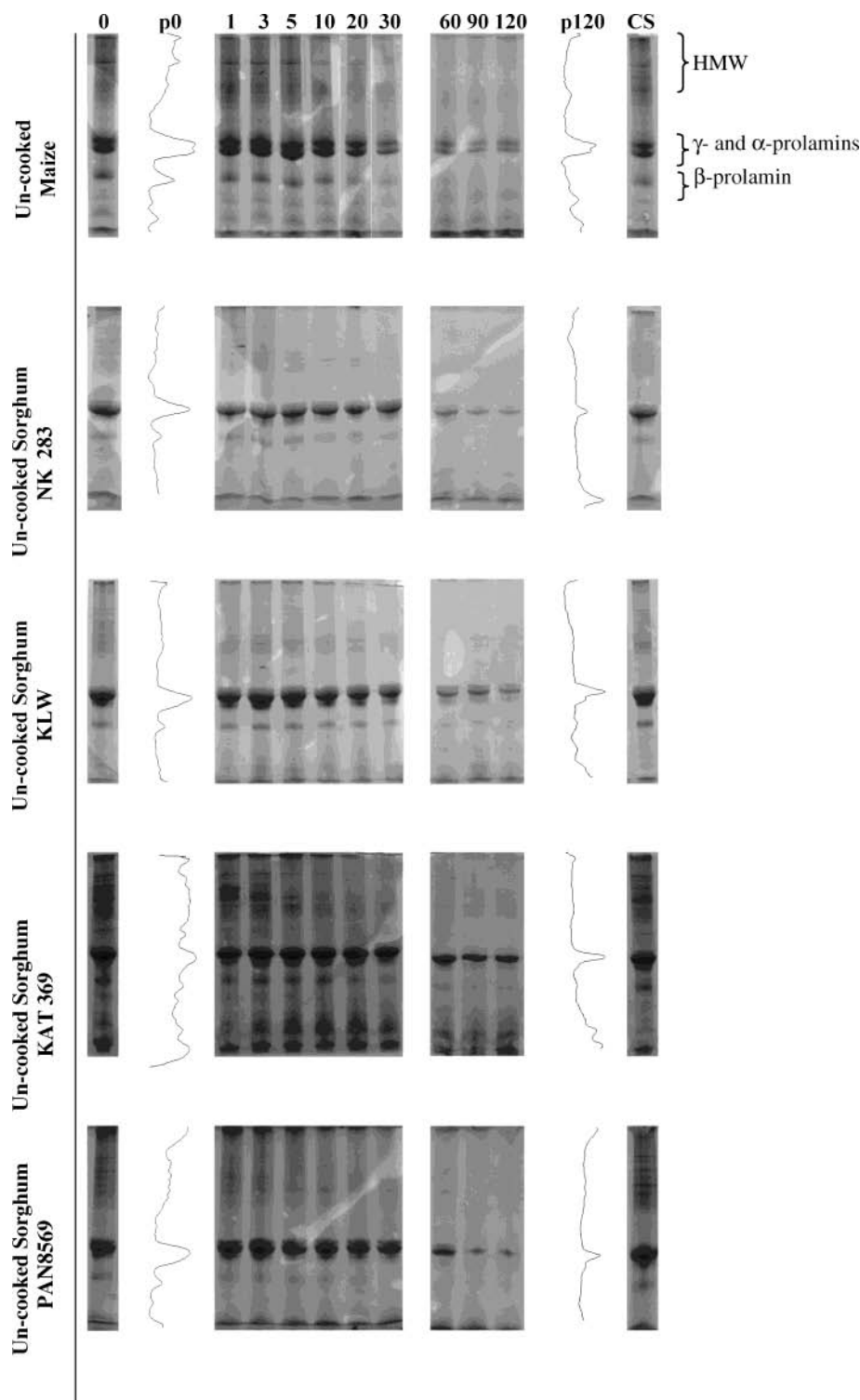
Figure 3. Electrophoretic gels corresponding to the sequential digestion of uncooked maize and sorghum samples. Next to the 0 and 120 digestion lanes are placed the corresponding electrophoretic profiles.

know if there are insoluble protein agglomerates left in the insoluble residue. To check the presence of protein in the SR fractions, their infrared spectra were acquired. The spectra show the starch features with no evident protein peaks. By PCA two main groups can be distinguished: the cooked and the uncooked samples (**Figure 1a**). As expected, the loadings profiles discriminate these two groups exclusively through fingerprint region, where starch absorbs (1100−900 cm^{-1}). No protein signs are responsible for the distinction of the samples, and no discrimination between digestion times is observed (**Figure 1b**).

Sorghum and Maize Protein Digestibility. Protein digestibility was defined as "the amount of protein digested by pepsin (20 mg of pepsin/mL of 0.1 M KH$_2$PO$_4$, pH 2, buffer) at 37 °C during 120 min". The nitrogen determination in UPR samples at 0 and 120 min was used to calculate the percentage of digestion of each cereal sample. The obtained results as percentage are shown in **Figure 2**.

Uncooked maize and sorghum present similar digestibility values, the digestibility from KLW being even higher than that
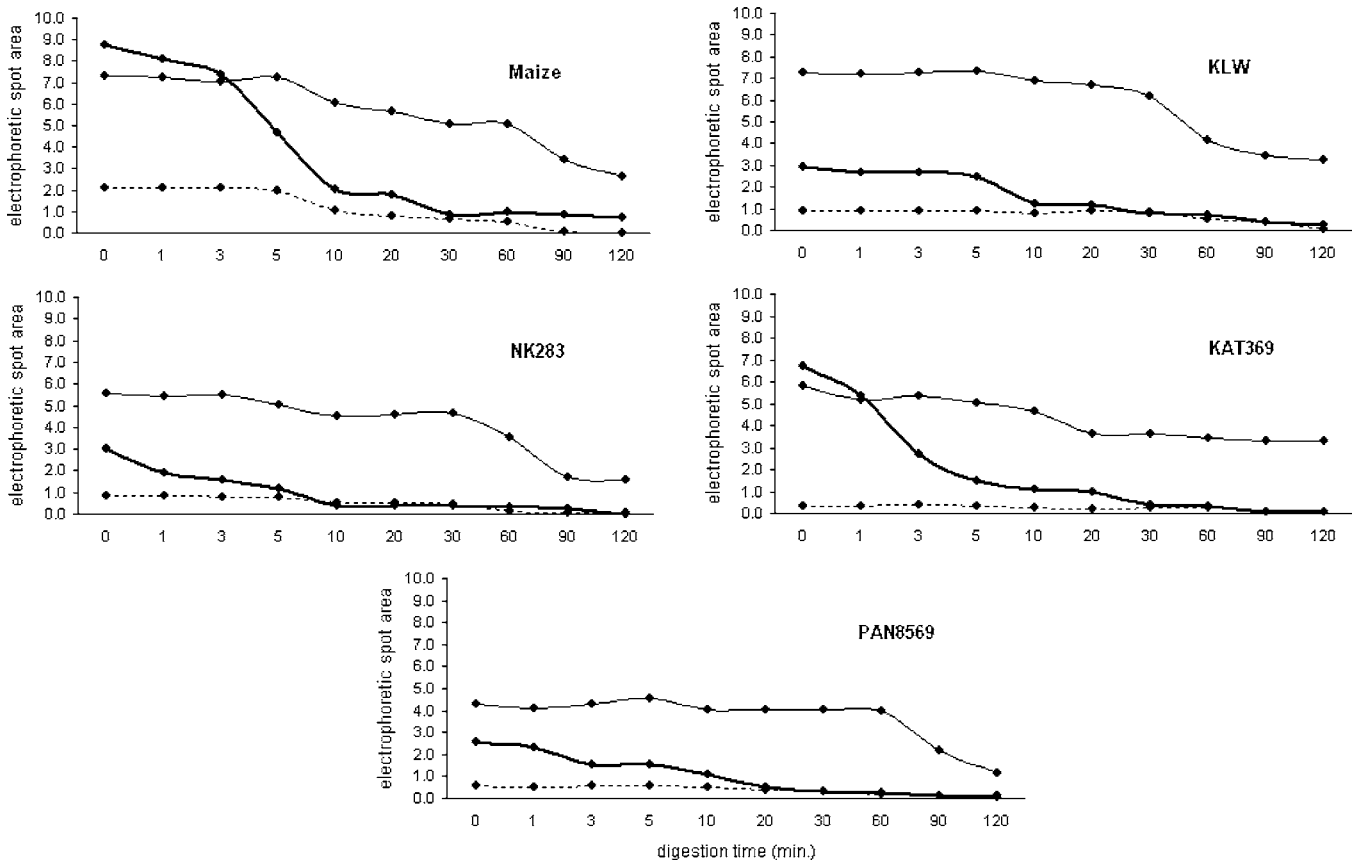
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Figure 4. Electrophoretic areas of HMW and monomers (γ- + α- and β-) during the 120 min of uncooked flour digestion.

from maize. This result is against the general assumption that sorghum is less digestible than maize.

Cooking, however, changes dramatically the digestibility values. As expected (*1*, *2*), maize is the less susceptible sample to a wet cooking procedure, the digestibility values likely being the same for uncooked and cooked samples. On the other hand, sorghum becomes less digestible by cooking, and among the sorghum samples KLW (which has the highest digestibility when not cooked) and KAT 369 are the more affected. In those varieties the percentages of decrease in protein digestibility are 56.58 and 52.28%, respectively. NK 283 and PAN 8564 sorghum samples present medium values with, respectively, 35.92 and 46.80% decreases in protein digestibility. As a consequence, varieties such as KLW and KAT 369 are not appropriate for cooking and should be used preferentially for fermentation or malting.

SDS-PAGE Study of the Sequential Digestion: Analysis of the UPE. Analyses of the electrophoretic gels of the cooked and uncooked UPE samples show that both maize and sorghum samples behave, in general, in similar ways.

Figure 3 shows that the HMW aggregates are the first proteins to be digested by pepsin in uncooked samples. **Figure 4** shows that the amount of HMW decreases from the beginning of the digestion and the decrease of monomers starts after just 5−10 min. These results are in agreement with previous publications which claim that the HMW proteins are present at the outer region of the protein bodies and consequently are more susceptible to pepsin (*11*). Our results confirm, as well, that β-kafirin is inside the protein body, as it is digested only at the same time as the γ- and α-kafirins.

For maize and NK 283, KAT 369, and PAN 8564 sorghums the γ-, α-, and β-monomer digestion begins after 20 min.

However, for KLW sorghum the monomer digestion is noticed after 10 min.

After 120 min, the β-prolamin from maize and NK 283 and PAN 8564 sorghums is completely digested, whereas for KLW and KAT 369 this monomer is still present at the end of the digestion.

Wet cooking increases the amount of high molecular aggregates in both maize and sorghum (**Figure 5**). This increase can be clearly noticed in the electrophoretic lane corresponding to the control sample (CS). In maize and sorhgum NK 283, a 45 kDa protein appears, and it remains until the end of the digestion. This protein, already reported in previous studies (*14*), resists both 120 min of pepsin digestion and the reducing conditions used in UPE extraction. In KLW, KAT 369, and PAN 8564 sorghum samples a second protein, resistant to digestion, appears in the 45 kDa region. This protein presenting a molecular weight of 47 kDa is reported here for the first time. These 45 and 47 kDa proteins are more evident in sorghum samples at 120 min of digestion time than in the corresponding maize sample.

The maize flour is the sample that presents the higher digestibility value and also presents fewer amounts of monomers in the electrophoretic lane corresponding to 120 min of pepsin digestion. As far as sorghum is concerned, the variety that shows higher digestibility values (NK 283) also presents fewer amounts of monomers in the 120 min lane. Sorghum samples with reduced protein digestibility (KLW, KAT 369, and PAN 8564) present higher amounts of monomers (γ-, α-, and β-) and two kinds of nonreducible proteins with 47 and 45 kDa.

The decrease in digestibility in sorghum could be associated with the formation of unreducible 47 and 45 kDa proteins. To

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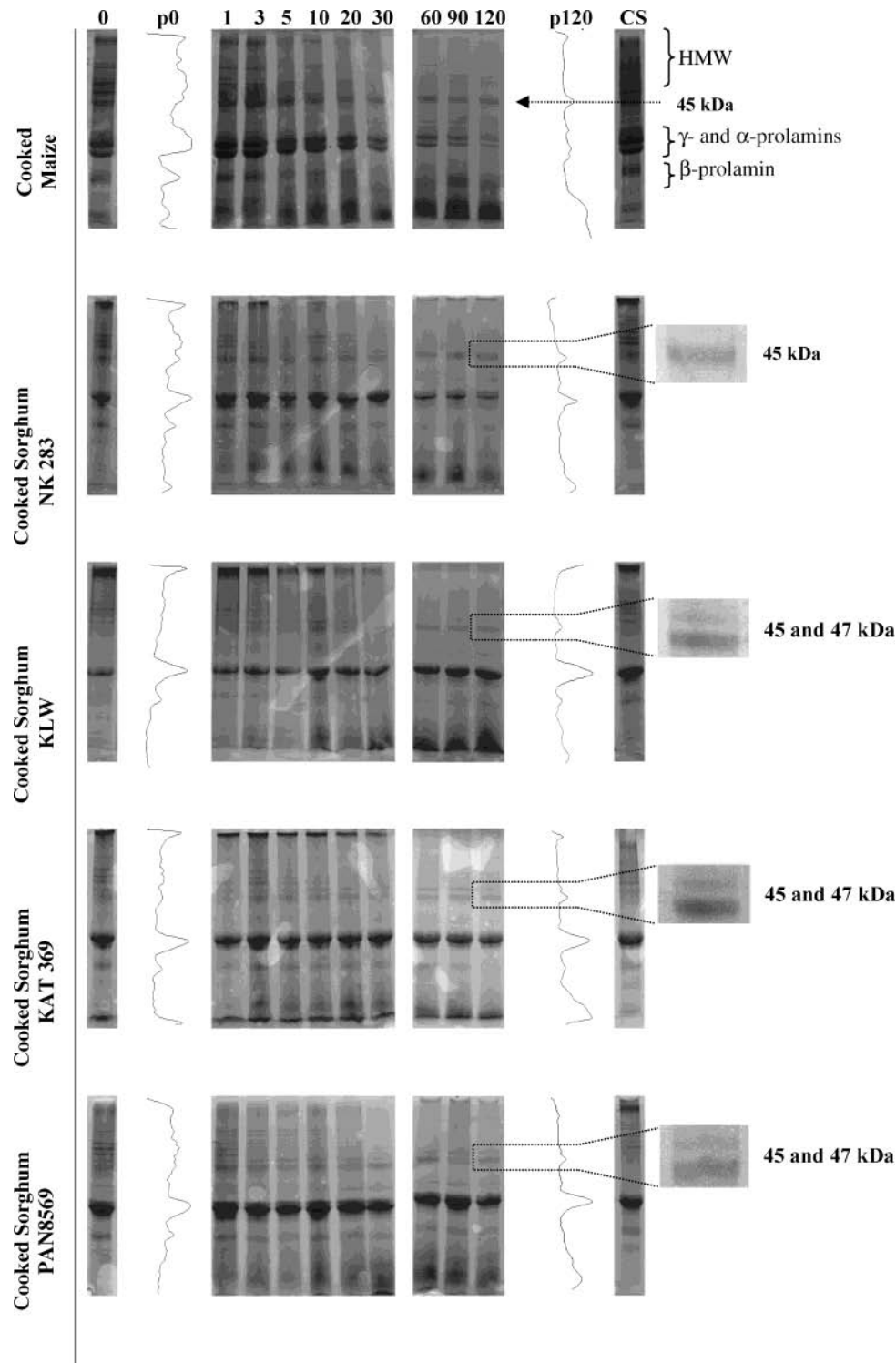
Figure 5. Electrophoretic gels corresponding to the sequential digestion of cooked maize and sorghum samples. Next to the 0 and 120 digestion lanes are placed the corresponding electrophoretic profiles.

what extent these proteins correlate with the reduction of digestion of the monomers is still under study.

Electrophoretic Spot Area versus Digestibility. To quantify the results described above, the electrophoretic spot areas from γ- and α-prolamins and from 45 and 47 kDa were measured for 0 and 120 min of digestion time. Those results are presented in **Figure 6**. The γ- and α-prolamin areas from uncooked samples show a reasonable decrease during digestion: 53.5% in maize, 71.5% in NK 283, 55.1% in KLW, 43.6% in KAT 369, and 62.5% in PAN 8569.

Reflecting the lower digestibility of the cooked sorghum samples, the corresponding areas of γ- and α-prolamin are less affected by pepsin. Decreases of 3.6, 2.5, and 5.9% were determined for KLW, KAT 360, and PAN 8569, respectively. It is interesting that for NK 283, the more digestible cooked sorghum, a higher decrease is noticed (29.1%) (**Figure 6a**).

Figure 6b presents the areas of 45 and 47 kDa fractions at 0 and 120 min. The presence of 45 and 47 kDa fractions in uncooked samples is negligible, and they disappear almost totally with digestion. In cooked samples the 45 and 47 kDa
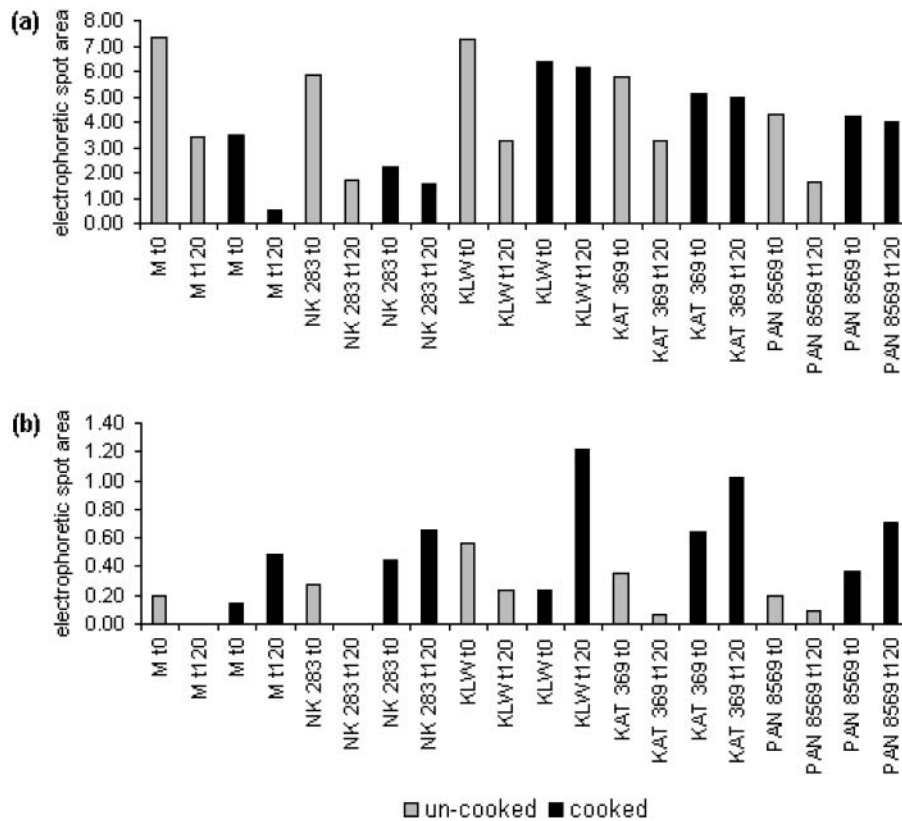
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Figure 6. (**a**) Electrophoretic spot areas of γ- and α-prolamins at 0 and 120 min of pepsin digestion and (**b**) electrophoretic spot areas of 45 and 47 kDa at 0 and 120 min of pepsin digestion, for both uncooked and cooked flour samples.

electrophoretic spots are much more evident with increasing digestion time. The areas at 120 min correlate with the loss of digestibility.

We noticed that both the resistance of γ- and α-prolamins to pepsin digestion and the increase of 45 plus 47 kDa fractions were useful to monitor the protein digestibility.

In summary, the decrease in protein digestibility upon cooking is different in the various varieties and does not correlate with the uncooked digestibility values. For instance, KLW is the most digestible flour when uncooked and is the most affected by cooking.

Sequential protein digestion of the uncooked flours showed that all sorghum varieties behave similarly; however, when flours are cooked, some major differences can be pointed out. The amount and type of the proteins that exist after 120 min of pepsin digestion are related to digestibility values. The less digestible the flour, the higher the remaining amounts of γ- and α-monomers and, particularly, 45 and 47 kDa proteins. The 47 kDa protein was not reported before and is present in cooked samples of the less digestible sorghum varieties (KLW, KAT 369, and PAN 8569). More detailed work has to be done to understand the formation of those resistant proteins.

ABBREVIATIONS USED

FT-IR, Fourier transform infrared; HMW, high molecular weight; M_r, molecular weight; PCA, principal component analysis; SDS-PAGE, sodium dodecyl sulfate–polycrylamide gel electrophoresis; SNV, standard normal variate; ST, starch residue; UPE, undigested protein extract; UPR, undigested protein residue.

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