ELSEVIER

Journal of Cereal Science 46 (2007) 22–31

Journal of CEREAL SCIENCE

www.elsevier.com/locate/yjcrs

Preferential binding of sorghum tannins with γ-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation

Janet Taylor[a], Scott R. Bean[b], Brian P. Ioerger[b], John R.N. Taylor[a,*]

[a]*Department of Food Science, University of Pretoria, Pretoria 0002, South Africa*
[b]*USDA-ARS, GMPRC, Manhattan, KS 66502, USA*

Received 17 May 2006; received in revised form 26 October 2006; accepted 1 November 2006

Abstract

Kafirins, sorghum prolamins bind with sorghum condensed tannins (CTs). The binding of different kafirin species with sorghum CTs was investigated. Analysis by chemical assay and by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), reversed-phase high-performance liquid chromatography (RP-HPLC), and free zone capillary electrophoresis (FZCE), showed that γ-kafirin bound more CTs than the other kafirin species. SDS–PAGE suggested that the γ-kafirin-bound tannins were in the form of aggregates of molecular size >200k. RP-HPLC and FZCE revealed that sample preparation and drying the kafirins prior to the binding assays had a significant impact on γ-kafirin solubility. The effect of tannin binding on kafirin and kafirin film digestibility and film biodegradation was determined. Kafirins bound to tannins had lower digestibilities than unbound kafirins. Films made from tannin-bound kafirin had much lower digestibility and were less biodegradable than films made from unbound kafirin. The increase in kafirin film life by tannin modification appears to be due to a decrease in protein digestibility caused by kafirin–tannin binding. These findings suggest that γ-kafirin content in sorghum may be manipulated to either reduce or increase tannin binding in order to change the functionality of the kafirin in food, feed or film applications.

Keywords: Sorghum; Tannin; Binding; Kafirin

1. Introduction

The properties of sorghum proteins, like those of other proteins, are influenced by interaction with tannins. This interaction is particularly important in the case of sorghum grain as a substantial proportion of varieties, the types II and III, contain condensed tannins (CTs) (reviewed by Awika and Rooney, 2004).

Interaction between tannins and sorghum proteins reduces both protein and starch digestibility (Serna-Saldivar and Rooney, 1995). This is important in both human (Salunkhe et al., 1990) and animal nutrition (Hancock, 2000). The formation of complexes between sorghum proteins and tannins is thought to render the proteins indigestible (Butler et al., 1984) as well as inhibit digestive enzymes (Nguz et al., 1998). During the brewing process sorghum malt amylase enzymes are inhibited by tannin interaction (Daiber, 1975). However, tannin–protein interactions can be used in some cases to improve protein functionality. For example, a useful application of sorghum tannin–protein interaction is the improvement in tensile properties of kafirin bioplastic films by tannin binding (Emmambux et al., 2004).

Proteins rich in proline bind more sorghum tannins than other proteins (reviewed by Spencer et al., 1988). In addition, a protein containing more proline repeats will bind more tannin than one with less such repeats (Baxter et al., 1997). Kafirin, the sorghum prolamin storage

Abbreviations: ANOVA, analysis of variance; BME, 2-mercaptoethanol; CT, condensed tannin; Dp, degree of polymerization; FZCE, free zone capillary electrophoresis; HPLC, high-performance liquid chromatography; PRP, proline-rich protein; RP-HPLC, reversed-phase high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

*Corresponding author. Tel.: +27 12 420 4296; fax: +27 12 420 2839.
E-mail address: jtaylor@postino.up.ac.za (J.R.N. Taylor).

0733-5210/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jcs.2006.11.001

protein, is relatively rich in proline (11 mole%) (Evans et al., 1987) and has been shown to form complexes with both sorghum CTs and tannic acid (Emmambux and Taylor, 2003). Kafirin is located in the sorghum grain starchy endosperm protein bodies. The different kafirin species are distributed in specific locations within the protein bodies. α-Kafirin, the major species is located in the central part of the protein body and β- and γ-kafirin are located at the protein body periphery or as inclusions with the protein body (Shull et al., 1992). γ-Kafirin is the most proline-rich of the kafirin polypeptide species (23 mole%) (reviewed by Shewry, 2002) and contains the most proline repeats (Swiss Prot, 2005) of the kafirins. In view of the importance of protein–tannin interactions in the nutritional and functional properties of sorghum, more in-depth knowledge of the interactions between kafirins and tannins are needed. Thus, the objective of this study was to determine whether γ-kafirin (due to its high levels of proline and proline repeats) preferentially binds to sorghum CT and to determine the effect of the tannin binding on kafirin *in vitro* digestibility.

2. Experimental

2.1. Materials

A mixture of two CT-free, tan plant, white sorghum cultivars PANNAR PEX 202 and 206 were used for total kafirin extraction. The method of total kafirin extraction was as described by Emmambux and Taylor (2003). γ-Kafirin was isolated from total kafirin using 0.05 M sodium lactate containing 2% (v/v) 2-mercaptoethanol (BME) (Evans et al., 1987) at a protein to solvent ratio of 1:5. Extraction was carried out for 3 periods of 1 h with constant stirring at 25 °C. After each period the mixture was centrifuged at 7200*g* for 10 min. The supernatant was removed and the pellet re-extracted for a further period. Supernatants were combined and dialyzed against distilled water over a period of 36 h with frequent changes of water. Dialyzed material and the residual pellet were freeze-dried and designated γ-kafirin and residual kafirin (i.e. total kafirin minus γ-kafirin), respectively. The identity of the γ-kafirin was confirmed by comparing its amino acid composition with that in the literature (Taylor and Belton, 2002) (Table 1). It was also confirmed using reversed-phase high-performance liquid chromatography (RP-HPLC) and free zone capillary electrophoresis (FZCE) by comparing the separation of the isolated γ-kafirin to previously published reports of the separation of the kafirin species (Bean et al., 2000).

CT was extracted using the method of Emmambux and Taylor (2003) from a red tannin sorghum (ex. Nola GH91) and used for the binding assay and preparation of tannin modified kafirin films. The CT content of the extract was 2730 mg/g (dry weight basis, catechin equivalents) determined by the ISO (1988) ferric ammonium citrate method.

Table 1
Amino acid composition (moles%) of kafirin preparations

Amino acid	Total kafirin	γ-kafirin	Residual kafirin
Asparagine	4.3	0.9 (0)	4.5
Glutamine	20.1	12.8 (11.9)	20.4
Serine	6.3	5.7 (5.2)	6.3
Glycine	3.2	9.4 (8.8)	2.7
Histidine	1.4	7.0 (7.8)	1.2
Arginine	1.4	2.7 (2.1)	1.2
Threonine	3.2	5.1 (4.7)	3.0
Alanine	15.1	6.7 (5.7)	15.5
Proline	11.1	18.7 (23.3)	10.7
Tyrosine	3.7	2.3 (2.1)	3.7
Valine	5.0	6.1 (6.2)	5.0
Methionine	1.1	1.4 (1.0)	1.2
Cystine	0.4	7.8 (7.8)	0.4
Isoleucine	4.1	2.7 (2.6)	4.2
Leucine	14.7	8.3 (8.3)	15.0
Phenylalanine	4.8	1.8 (1.6)	4.9
Lysine	0.2	0.6 (0)	0.2

Figures in parenthesis are literature values from Taylor and Belton (2002).

3. Methods

3.1. Amino acid analysis

The amino acid composition of the protein preparations was determined, using the Pico-Tag method (Bidlingmeyer et al., 1984) and analyzed by RP-HPLC.

3.2. Tannin binding assay

The method of Emmambux and Taylor (2003) was used with slight modification. Total kafirin, γ-kafirin and residual kafirin (1 mg protein (N × 6.25)/ml) and CT (0–400 μg/ml) (in terms of catechin equivalents) were dissolved individually in 75% (v/v) absolute ethanol containing 0.02 M (pH 4.0) phosphate buffer. One milliliter kafirin solution was mixed with 1 ml CT solution and incubated for 1 h at 60 °C in a shaking waterbath. Samples were chilled overnight at 4 °C before being vortexed and centrifuged at 2000*g* for 5 min. The supernatant was then decanted and used for the determination of residual CT. The amount of CT bound to protein was determined by difference between the total amount of CT added and the amount remaining, as it was not possible to completely re-solubilize the precipitated CT-bound protein. The ISO (1988) ferric ammonium citrate method was used to quantify tannins using catechin (Sigma, St. Louis, MO, USA) as a standard.

Samples for determination of protein digestibility and characterization by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were prepared using a 10 mg/ml protein solution and a 23 mg/ml CT solution. After binding as described above and chilling overnight, samples were centrifuged at 2000*g* for 15 min. The supernatant was retained and the pellet washed with the ethanol/

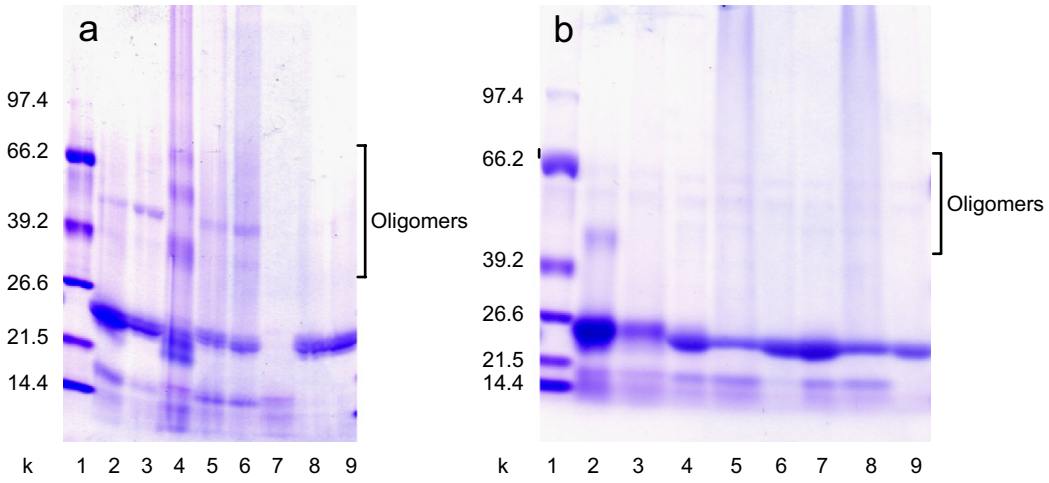

Fig. 1. SDS–PAGE of kafirin species and kafirin species bound to sorghum condensed tannins (CT) under non-reducing (A) and reducing conditions (B) (A) 1—molecular weight standards, 2—total kafirin, 3—residual kafirin, 4—γ-kafirin, 5—total kafirin bound to CT, 6—residual kafirin bound to CT, 7—γ-kafirin bound to CT, 8—supernatant total kafirin bound to CT, 9—supernatant residual kafirin bound to CT (B) 1—molecular weight standards, 2—γ-kafirin, 3—γ-kafirin bound to CT, 4—total kafirin, 5—total kafirin bound to CT, 6—supernatant total kafirin bound to CT, 7—residual kafirin, 8—residual kafirin bound to CT, 9—supernatant residual kafirin bound to CT.

buffer solution. This was repeated twice more to remove any unbound CT and the supernatants were bulked. Ethanol was allowed to evaporate off at room temperature before the supernatants and pellets were freeze-dried. The supernatant was assumed to contain any remaining unbound protein as shown by SDS–PAGE (Fig. 1(A), tracks 8 and 9 and Fig. 1(B), tracks 6 and 9), whereas the pellet contained the precipitated, insoluble tannin–protein complexes.

3.3. SDS–PAGE

Protein preparations were characterized by SDS–PAGE on a 4–18% acrylamide gradient both under reducing and non-reducing conditions. A gradient system was used in order to separate high-molecular-weight oligomers present in the samples. The gradient was prepared from a stock of 40% (w/v) acrylamide/bis (19:1) (Merck, Halfway House, South Africa) and allowed the separation of proteins with a M_r of 14–200k. A vertical electrophoresis system (Protean II, Bio-Rad, Hercules, USA) was used with gels of 140 mm length and 1.5 mm thick according to Gallagher (1999). The different protein preparations were loaded to constant protein ($\approx$15 μg) as determined by the Dumas combustion method (AACC Standard Method 46-30, 2000). Special care was taken to completely resolubilize the CT-bound samples. This was done by repeatedly heating and vortexing until solubilization was obtained. Molecular weight markers (low-range protein marker, Roche Molecular Biochemicals, Indianapolis, IN, USA) were used. Proteins were stained with Coomassie Brilliant Blue R250.

3.4. RP-HPLC and FZCE

RP-HPLC and FZCE were applied to investigate kafirin–tannin binding in more detail, as described by Bean et al. (2000). Binding of tannins to purified kafirins was carried out as described above, except 2% BME was included in the binding solvent and an ammonium acetate buffer was used in place of the sodium phosphate buffer. Kafirin was isolated from aqueous ethanol extracts containing sodium metabisulfite as described in Bean et al. (2006). Four levels of CT (0%, 5%, 10%, and 20% w/w) were tested.

To investigate the effects of drying protein isolates on tannin–kafirin interactions, isolated kafirins were mixed with CT (at 0%, 5%, and 20% w/w) as described above. After incubating for 1 h, aliquots were removed and dried under various conditions. For the control treatment, samples were analyzed by RP-HPLC immediately upon completion of the binding assay. For the test treatment, aliquots were taken to dryness using heat by placing the samples in a heated vacuum chamber with the centrifugal sample chamber heated using an instrument setting of medium (air temperature of the chamber was ~40 °C). In a further treatment, aliquots were lyophilized to dryness. All dried samples were subsequently resolubilized in the solvent used for the binding assay and then analyzed by RP-HPLC.

3.5. Film formation

Total kafirin, kafirin bound to CT (20% with respect to protein) and zein (Sigma Z-3625, St. Louis, MO, USA) were used to cast free standing films as described by Emmambux and Taylor (2003).

3.6. Protein digestibility of protein preparations and films

The pepsin method of Hamaker et al. (1987) was used. Protein preparations and films (10 mg protein basis) were digested for 2 h at 37 °C with 998 units of pepsin P7000

(Sigma). Total protein and residual protein (N × 6.25) was determined by the Dumas combustion method (AACC Standard Method 46-30). Protein digestibility was calculated by the difference between the total protein and the residual protein after pepsin digestion divided by the total protein and expressed as a percentage.

3.7. Film biodegradation

Biodegradation of films was determined using a procedure based on ASTM Method D5512-96 (ASTM 1996). Films were cut and mounted into 35 mm plastic film slide frames and imaged using a flatbed scanner. The mounted films were then buried in well-fermented compost (horse manure and sawdust, moisture 37% or 60%) contained in plastic buckets with tight fitting lids. The buckets were incubated in the dark at 35 °C for 2 days, 58 °C for 4 days, 50 °C until day 26 for optimal composting conditions and 35 °C for the final 2 days of the test. The containers were aerated daily by removing the lids for 1 h. Films were removed at 5-day intervals, cleaned gently with a soft brush and scanned. A low-density polyethylene film was included for comparison.

3.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine whether there was a significant ($P < 0.05$) effect of kafirin type on the amount of CT bound and whether there were any significant differences in protein digestibility between protein type, whether the protein was bound and between different film types.

4. Results and discussion

4.1. Kafirin–tannin binding

Isolated γ-kafirin bound the most CT (70–77%), whereas total kafirin and residual kafirin bound similar, but far lower, amounts of CT, 35–45% and 35–40%, respectively (Fig. 2). The fact that the percentage CT bound to the kafirin species remained the same from 10% to 40% of CT added with respect to kafirin implies that the kafirin was saturated with CT, similar to that found by Emmambux and Taylor (2003).

With regard to the relative amount of CT bound by the different kafirin species several factors may be involved, including tannin composition. Awika et al. (2003) using a similar tannin extraction procedure found that the procyanidin profiles of two sorghum varieties were 14–31% oligomers of degree of polymerization (dp) 1–10 and the remainder were oligomers larger than dp 10. It is expected that the profile of the CT extract used in this study was similar. Since the same CT extract was used to bind each of the kafirin species the degree of protein–tannin binding would only be affected by the type of kafirin bound. Charlton et al. (2002) showed that intermolecular

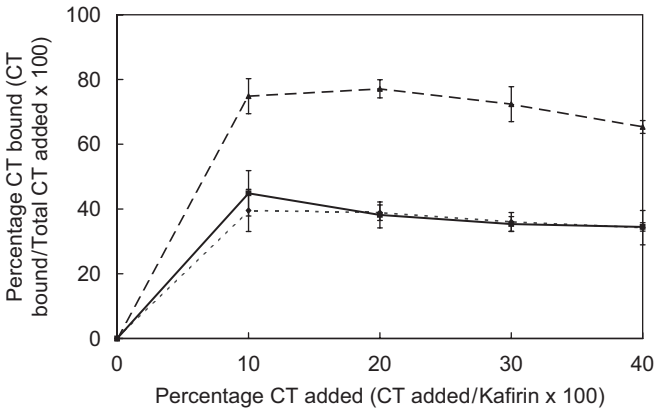

Fig. 2. Percentage of sorghum condensed tannin (CT) bound to different kafirin species. Long dashes: γ-kafirin, Solid line: Total kafirin, Short dashes: Residual kafirin (kafirin after γ-kafirin removed).

binding between peptides and tannins is dominated by the stacking of polyphenolic rings onto planar hydrophobic surfaces. This binding is strengthened by multiple binding of polyphenolic rings. Effectively the peptide becomes increasingly coated with polyphenol until polyphenol bridges occur and the peptide dimerizes and precipitates out. Since up to 40% CT relative to the kafirins was added it would be expected that the precipitated CT–kafirin material would consist of very large molecular weight aggregates of kafirin polymers and CT molecules.

It has long been known that proteins containing high amounts of proline bind more sorghum tannins than those with less proline (reviewed by Spencer et al., 1988). Thus the fact that γ-kafirin bound most CT is not surprising since it contains far more proline (18.7 mole%) than the total kafirin (11.1 mole%) or residual kafirin (10.7 mole%) (Table 1). Another important factor that determines the amount of tannin bound to protein is the number of proline repeats that the protein contains (Baxter et al., 1997). γ-Kafirin has three repeats of two proline residues, three repeats of three residues, and one repeat of four residues (Swiss Prot, 2005). This would be expected to give γ-kafirin a more open structure than total kafirin, which is primarily α-kafirin and in its native form is mainly α-helical (Gao et al., 2005). According to Butler et al. (1984) proline residues disrupt the α-helix, breaking internal hydrogen bonds and so provide an opportunity for forming multiple hydrogen bonds between tannin molecules and the peptide backbone and to also maximize non-polar interactions. The side chain of proline is a five-membered pyrrolidine ring structure. As a result of this structure, proline residues cause the peptide chains to form a rigid and extended conformation (Edens et al., 2005). Multiple proline residues give rise to a higher degree of rigidity and allow both hydrogen bonds and hydrophobic interactions to form between peptide chains and tannins. It may have been expected that residual kafirin would bind less CT than total kafirin since the γ-kafirin had been removed. This was not so (Fig. 2), possibly because the γ-kafirin represents only a

small proportion (9–12%) of the total kafirin (Shewry, 2002). This is also reflected by the fact that the proline content of the residual kafirin (10.7 mole%) was found to be virtually the same as that for total kafirin (11.1 mole%) (Table 1).

SDS–PAGE under non-reducing conditions (Fig. 1(A)) of the CT-bound γ-kafirin (track 7) showed a band of 14–18k possibly equivalent to the 16k band of γ-zein shown by Kim et al. (2002). The additional bands of 27 or 50k for γ-zein observed by Kim et al. (2002) were not seen for γ-kafirin. In contrast, the unbound γ-kafirin (track 4) showed 14–18k bands plus strong bands at 22–24k, plus bands at 39, 60 and 74k, the latter three presumably being disulfide linked γ-kafirin dimers, trimers and tetramers. However, under reducing conditions (Fig. 1(B)) unbound γ-kafirin (track 2) showed the expected strong band of slightly higher apparent molecular weight (Shewry, 2002) than the total kafirin (track 4), which is mainly α-kafirin (Shewry, 2002). Under reducing conditions (Fig. 1(B)) CT-bound γ-kafirin (track 3) gave a much fainter band that the unbound γ-kafirin (track 2). This is evidence that much of the CT-bound γ-kafirin was in the form of aggregates of molecular size >200k that were too large to enter into the separating gel. Sarni-Manchado et al. (1999) working with grape seed tannins bound to salivary proline-rich protein (PRP) obtained similar results in that bound material did not enter the electrophoresis separating gel, whereas the unbound material did.

With regard to tannin binding by the total kafirin and residual kafirin, the intensity of the CT-bound total kafirin (Fig. 1(A) track 5 and Fig. 1(B) track 5) and CT-bound residual kafirin (Fig. 1(A) track 6 and Fig. 1(B) track 8) was somewhat reduced compared to the unbound protein species both under non-reducing and reducing conditions (Figs. 1(A) and (B), total kafirin tracks 2 and 4 and residual kafirin tracks 3 and 7, respectively), but not nearly to the same extent as with the γ-kafirin. This finding is in agreement with their lower level of tannin binding compared to γ-kafirin (Fig. 2). In this regard, there was some protein in the supernatant recovered when total kafirin and residual kafirin were bound to CT, but not with γ-kafirin. The amount of this material was not quantified. However, SDS–PAGE showed that it was mainly α-kafirin (Fig. 1(A) tracks 8 and 9, and Fig. 1(B) tracks 6 and 9, total and residual kafirin under reducing and non-reducing conditions, respectively). It is probable that the majority of this material was kafirin, which did not bind to the CT, this was reflected by the higher intensities of these bands in comparison with the CT bound kafirins.

To further investigate the binding of kafirins to CT, samples of kafirin–CT mixtures were separated by RP-HPLC (Fig. 3) and FZCE (Fig. 4). Both RP-HPLC and FZCE separations revealed that as the level of CT added to the protein extracts increased, the amount of γ-kafirin present decreased (Fig. 5), with roughly an 85% difference between the control (no CT) and 5% CT levels. The

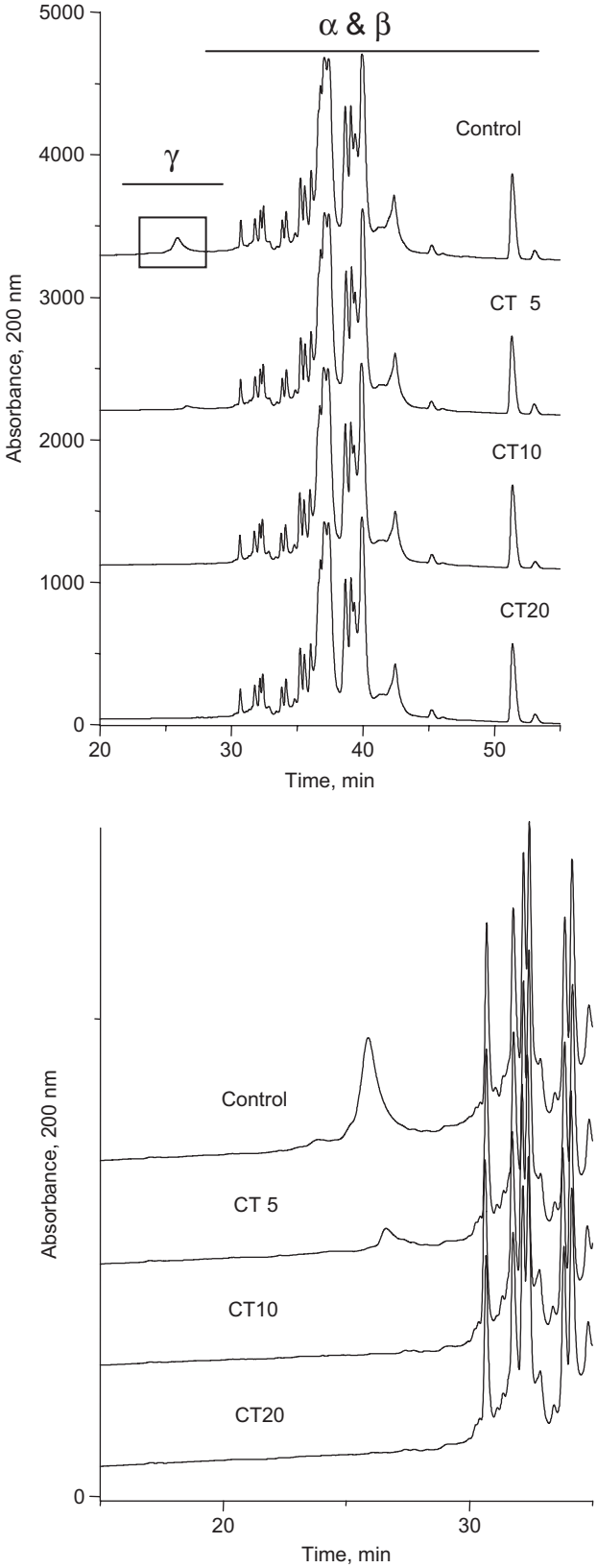

Fig. 3. RP-HPLC separations of isolated kafirins after being mixed with various levels of condensed tannin (CT). Location of kafirin subclasses are indicated on the figure. Boxed area is shown in expanded scale on the lower chromatogram.

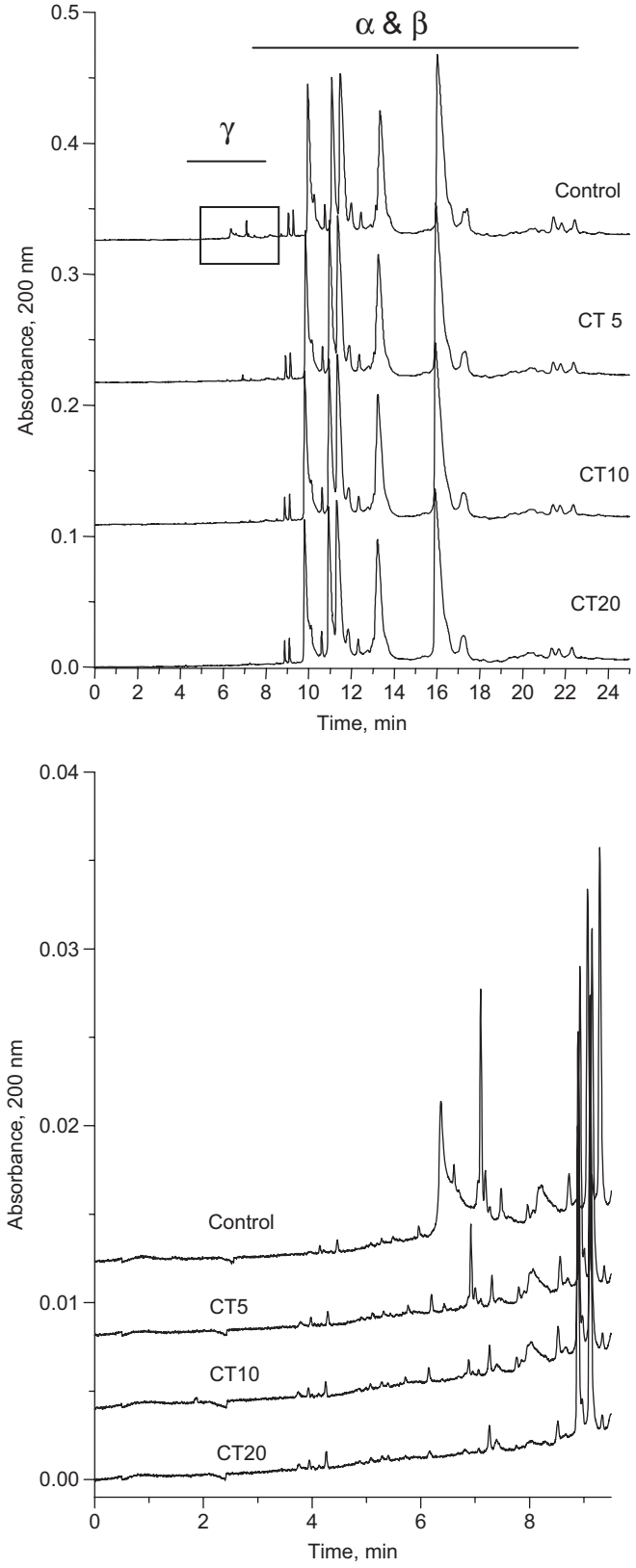

Fig. 4. FZCE separations of isolated kafirins after being mixed with various levels of condensed tannin (CT). Location of kafirin subclasses are indicated on the figure. Boxed area is shown in expanded scale on the lower electrophoregram.

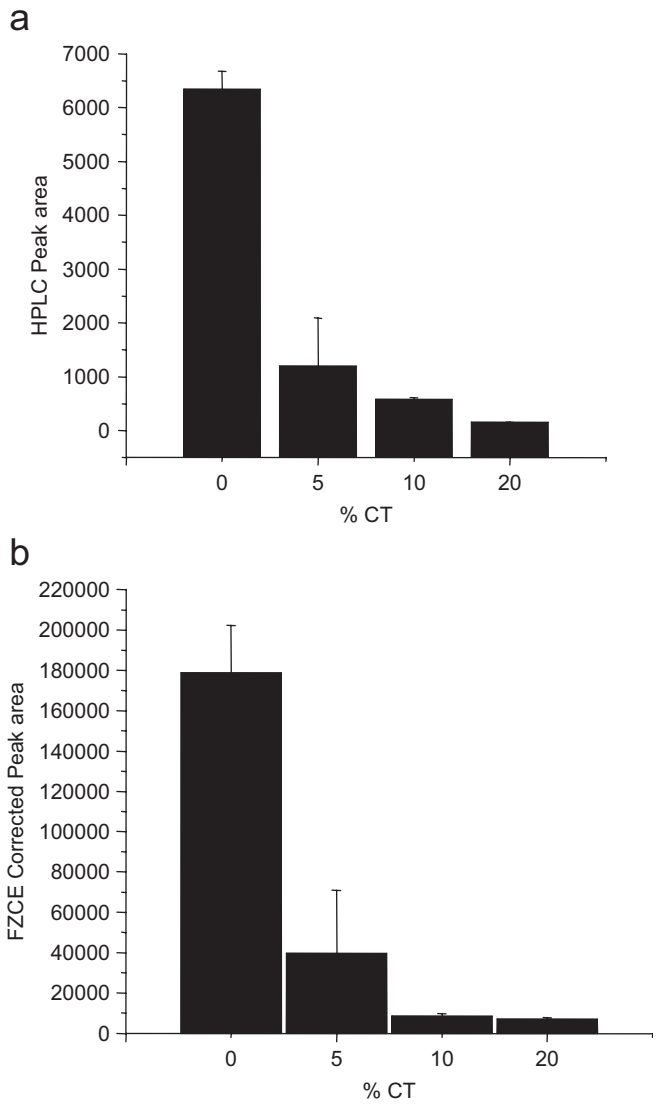

Fig. 5. Amount of γ-kafirin present in (A) RP-HPLC separations and (B) FZCE separations after kafirins were incubated with various levels of condensed tannin (CT). Error bars represent standard deviations ($n = 2$).

disappearance of the γ-kafirin in both the RP-HPLC and FZCE in the presence of CT supports the conclusion that CT preferentially binds γ-kafirin. The γ-kafirin bound to CT form insoluble complexes as mentioned above and thus are not seen in the RP-HPLC and FZCE separations. Furthermore, as the level of CT increased, the amount of γ-kafirin decreased, but no changes were seen in the α- and β-kafirin peak areas (Fig. 6). Thus, even at relatively high levels of CT (20% w/w), α- and β-kafirins were still not effectively bound and rendered insoluble by CT. No evidence for the formation of soluble complexes between CT and kafirins was seen in the RP-HPLC or FZCE separations. It would be expected that soluble protein–CT complexes would separate differently from non-bound kafirins. However, the RP-HPLC and FZCE patterns of samples mixed with CT were identical to those not mixed with CT.

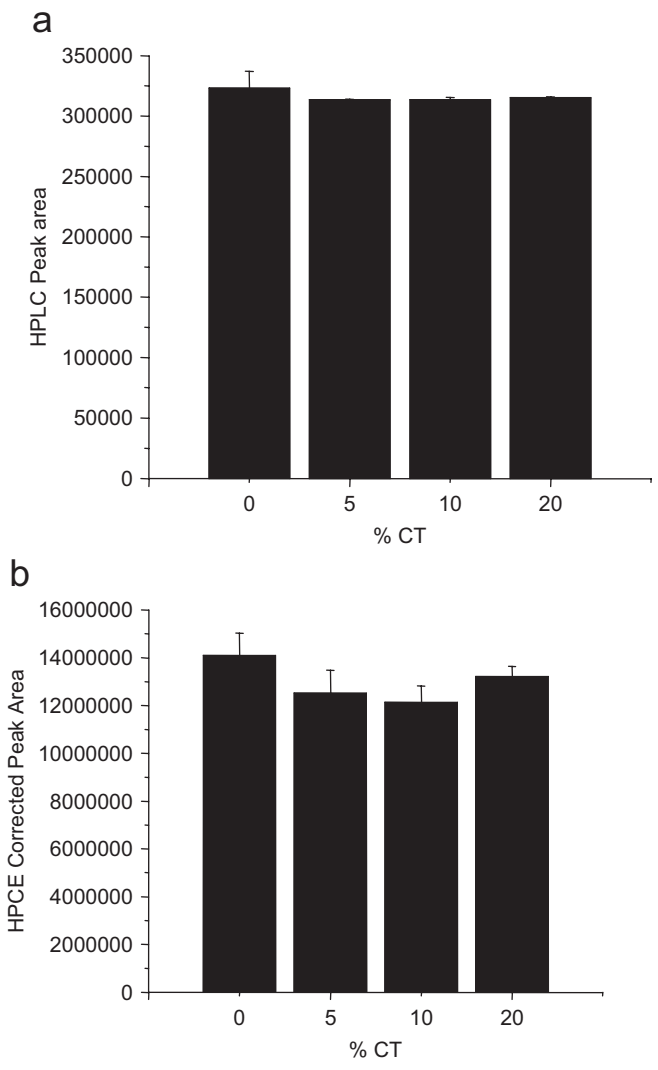

Fig. 6. Amount of α- and β-kafirin present in (A) RP-HPLC separations and (B) FZCE separations after kafirins were incubated with various levels of condensed tannin (CT). Error bars represent standard deviations ($n = 2$).

In preliminary research, kafirin–CT complexes were taken to dryness before subsequent analysis. Experiments were carried out to determine whether this could affect the interaction between tannins and proteins. When both heat dried and lyophilized samples were analyzed, reduced levels of γ-kafirins were seen in the chromatograms of the controls (no CT added) (Fig. 7). As expected from the above results, when kafirins were mixed with CT, γ-kafirin disappeared from the chromatograms. It is clear that drying the samples had an effect on the recovery of the γ-kafirin. Gao et al. (2005) reported changes to kafirin proteins during extraction and drying corresponding to the formation of β-sheet structures; an indication of protein aggregation. These authors also reported that the type of drying and the original extraction solvent of the proteins had an impact on the amount of β-sheet structures formed during drying and consequently on the solubility of the dried samples. In protein solubility tests, samples with higher amounts of β-sheet structures had poorer solubility compared to those with less β-sheet structures. Thus, aggregation and formation of β-sheet structures may be the reasons that γ-kafirins were poorly recovered when the samples were dried after the binding assay was conducted. However, there was no evidence that drying the kafirin together with the CT forced interactions with the tannins, as levels of α- and β-kafirins remained constant in the samples dried in the presence of CT (Fig. 7).

During the course of the RP-HPLC and FZCE separations, it was found that the solvents used to resuspend dried CT–kafirin mixtures affected the amount of γ-kafirin recovered. For example, when no reducing agent was used in the binding assay solvent, lower levels of γ-kafirin were seen in the analytical separations (data not shown). Also, the use of 60% *t*-butanol in the binding assay solvent (in place of 75% ethanol) resulted in higher levels of γ-kafirin seen in subsequent separations (data not shown). This was probably due to the less polar *t*-butanol being more effective at disrupting the protein aggregation caused during drying than ethanol. The inclusion of reducing agents in the solvent would probably reduce disulfide bonds formed during drying and thus likewise aid protein solubility. It should be noted that the isolated kafirins used in the binding assays for RP-HPLC and FZCE (shown in Figs. 3 and 4) displayed good recovery of γ-kafirin, as did the samples prepared for SDS–PAGE. Both methods used to prepare these proteins used a number of purification steps and isolation of proteins by precipitation and dialysis before being lyophilized (not heat dried). The samples used for the data shown in Fig. 7 were initially isolated using the same procedures, but then solubilized and dried again which obviously reduced the subsequent recovery of the proteins. Thus, sample preparation should be an important consideration when preparing samples for investigating tannin–kafirin interactions.

4.2. Effect of tannin binding on kafirin digestibility

When the kafirin species were bound to CT their digestibilities were reduced (Table 2). Kafirins bound to CT were far less digestible than unbound kafirins. Kafirin coated with a layer of tannins in the manner described by Charlton et al. (2002) would be expected to be less accessible to pepsin than unbound kafirin. It is of interest that unbound γ-kafirin was much less digestible than either total kafirin or residual kafirin (Table 2). This may be due to the γ-kafirin existing primarily in the form of disulfide cross-linked oligomers, as shown by SDS–PAGE (Fig. 1). Disulfide cross-linking of kafirins has been shown to reduce protein digestibility (Duodu et al., 2002).

When bound with CT, γ-kafirin as insoluble CT γ-kafirin complexes were slightly more digestible than either the total kafirin or residual kafirin CT insoluble complexes (Table 2). It is not clear why this is so. It is speculated that the bound γ-kafirin may have a slightly more open structure due to the proline repeats it contains, as described

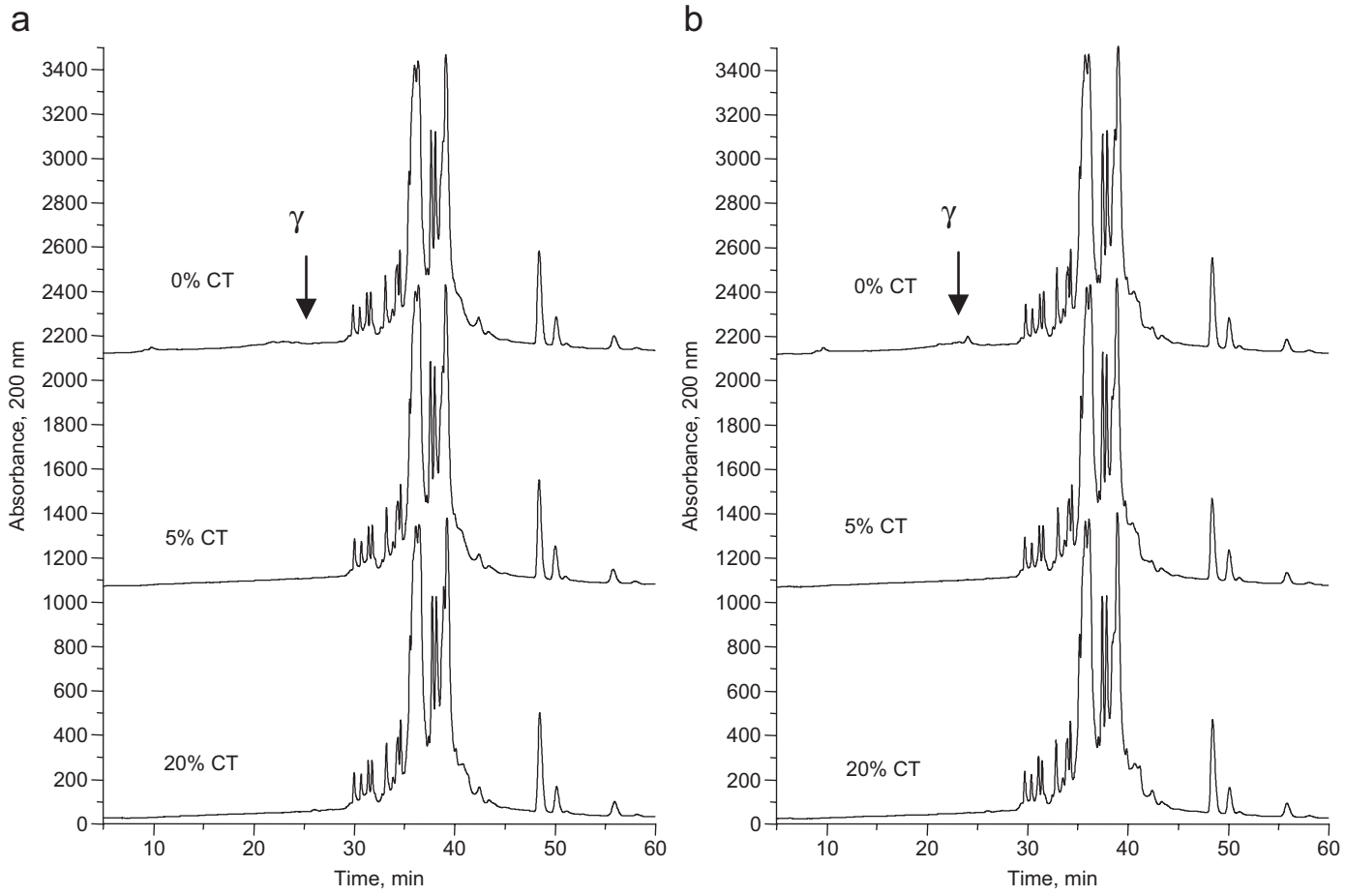

Fig. 7. RP-HPLC separations of kafirins after being mixed with various levels of condensed tannin (CT) and (A) lyophilized or (B) heat dried.

Table 2
In vitro protein digestibility[a] (%) of sorghum condensed tannin (CT) bound and unbound kafirin preparations and of films

Sample	Unbound	CT bound
Protein		
Total kafirin	59.1e (1.8)	8.4ab (1.2)
γ-kafirin	38.0d (2.1)	17.2c (1.9)
Residual kafirin	61.2e (3.9)	7.6a (1.8)
Films		
Total kafirin	41.5y (2.2)	16.7x (1.2)
Zein	54.4z (3.9)	Not determined

Values with different letters in a block are significantly different at the 95% level.
Protein and film data statistically analyzed separately.
[a]Each value represents the mean of four replicate analyses with standard deviation in brackets.

above, than bound total kafirin or bound residual kafirin. Duodu et al. (2003) reviewing sorghum protein digestibility refers to the importance of protein conformation and the extent to which the enzyme has accessibility to the protein on the determination of a protein's digestibility.

As expected, films made from CT-bound kafirin had much lower protein digestibility than films made from unbound kafirin (Table 2). The latter were slightly less digestible than the zein films. The kafirin used for film formation contained α- β-, and γ-kafirin (Fig. 1). β- and γ-kafirin would be capable of forming disulfide cross-linkages during film formation because of their high cysteine content. The zein films were made from commercial zein, which consists of mainly α-zein (Lawton, 2002) and would be less able to form disulfide linkages because of its lower amount of cysteine residues. Hence, the kafirin structure would be less accessible to pepsin digestion than the zein, resulting in lower protein digestibility of the kafirin films than the zein films. Also of interest was the fact that kafirin films were somewhat less digestible than the kafirin they were made from Table 2. This is contrary to the findings of Byaruhanga et al. (2005), where kafirin films were much more digestible than the kafirin they were made from. This difference was probably due to the different casting solvents used. Byaruhanga et al. (2005) used glacial acetic acid as opposed to aqueous-ethanol in this work. Glacial acetic acid was thought to deamidate the

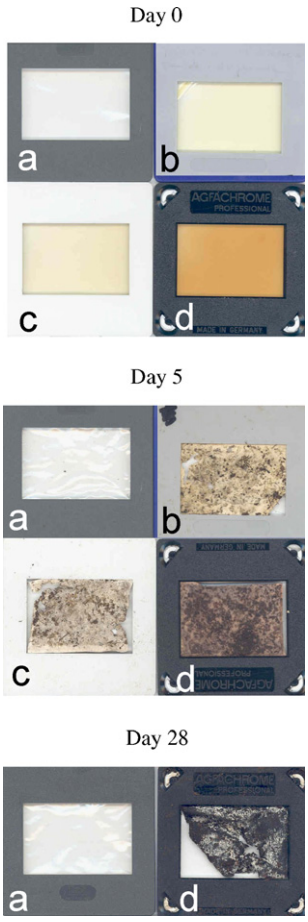

Fig. 8. Biodegradation of films under high moisture conditions: (a) LDPE, (b) zein film, (c) total kafirin film, and (d) film made with kafirin bound to CT.

glutamine residues of the kafirin resulting in breakage of hydrogen bonds, making the film readily accessible to pepsin digestion (Byaruhanga et al., 2005). Casting the kafirin films in aqueous ethanol at elevated temperatures, as in this study, would promote the formation of disulfide linkages, as previously discussed, resulting in the formation of less digestible kafirin oligomers.

Films made from unbound kafirin were biodegradable under aerobic conditions within 20 days under low moisture conditions and within 10 days under high moisture conditions (Fig. 8), a similar time to zein films. Films made from CT-bound kafirin took up to 8 days longer to biodegrade under both high and low moisture conditions. Degradation under low moisture conditions followed a similar pattern but the degradation was slower (not shown). This was probably due to greater microbial growth and subsequent metabolism at the higher moisture level. Film degradation followed a similar pattern for all films. As time progressed they became opaque and wrinkled. Subsequently, the films developed pin-holes, which progressively became larger as the film fragmented and finally completely degraded. The extended life of films made from CT-bound kafirin is probably related to their lower protein digestibility. Since binding with CT reduced film protein pepsin digestibility, it would also be expected to reduce microbial degradation due to a lower accessibility of the proteins to the microbial hydrolytic enzymes. Inducement of protein cross-linkages has also been reported to slow biodegradation of heat-treated whey protein films (Le Tien et al., 2000), γ-irradiated soya protein films (Lacroix et al., 2002) and heat-treated kafirin films (Byaruhanga et al., 2005).

5. Conclusions

γ-Kafirin binds the most CT compared to the α- and β-kafirin, probably due to its high proline content. The protein digestibility of kafirin–tannin complexes is much lower than unbound kafirins. However, kafirin films made with bound tannins appear to have extended life due to a decrease in protein digestibity caused by kafirin–tannin binding. Thus, to improve protein digestibility of sorghum food and feed, an approach could be to breed for sorghum lines with reduced or even no γ-kafirin, as is being done in the Africa Biofortified Sorghum project (www.supersorghum.org). Conversely, to produce kafirin bioplastics, such as films, with increased shelf-life, kafirin rich in γ-kafirin should be used in conjunction with modification by tannins. Additionally, sorghum foods with high γ-kafirin and high tannin could be a mechanism of lowering calorie intake and reducing the incidence of obesity.

References

American Association of Cereal Chemists, 2000. Crude Protein-Combustion, Standard Method 46-30. Approved Methods of the AACC. The Association, St. Paul, MN.

Awika, J.M., Dykes, L., Gu, L., Rooney, L.W., Prior, R.L., 2003. Processing of sorghum (*Sorghum bicolor*) and sorghum products alters procyanidin oligomer and polymer distribution and content. Journal of Agricultural and Food Chemistry 51, 5516–5521.

Awika, J.M., Rooney, L.W., 2004. Sorghum phytochemicals and their potential impact on human health. Phytochemistry 65, 1199–1221.

ASTM, 1996. Annual Book of the American Society for Testing and Materials Standards. D5512-96. Standard Practice for Exposing Plastics to a Simulated Compost Environment using an Externally Heated Reactor. The Society, West Conshohocken, PA.

Baxter, N.J., Lilley, T.H., Haslam, E., Williamson, M.P., 1997. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. Biochemistry 36, 5566–5577.

Bean, S.R., Lookhart, G.L., Beitz, J.A., 2000. Acetonitrile as a buffer additive for free zone capillary electrophoresis separation and characterization of maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* L. Moench) storage proteins. Journal of Agricultural and Food Chemistry 48, 318–327.

Bean, S.R., Ioerger, B.P., Park, S.H., Singh, H.S., 2006. Interaction between sorghum protein extraction and precipitation conditions on yield, purity, and composition of purified protein fractions. Cereal Chemistry 83, 99–107.

Bidlingmeyer, B.A., Cohen, S.A., Tarvin, T.L., 1984. Rapid analysis of amino acids using pre-column derivatization. Journal of Chromatography 33 (6), 93–104.

Butler, L.G., Riedl, D.J., Lebryk, D.G., Blytt, H.J., 1984. Interaction of proteins with sorghum tannins: Mechanism, specificity and significance. Journal of the American Oil Chemists Society 6, 916–920.

Byaruhanga, Y., Erasmus, C., Taylor, J.R.N., 2005. Effect of microwave heating of kafirin on the functional properties of kafirin films. Cereal Chemistry 82, 565–573.

Charlton, A.J., Baxter, N.J., Lockman Khan, M., Moir, A.J.G., Haslam, E., Davis, A.P., Williamson, M.P., 2002. Polyphenol/peptide binding and precipitation. Journal of Agricultural and Food Chemistry 50, 1593–1601.

Daiber, K.H., 1975. Enzyme inhibition by polphenols of sorghum grain and malt. Journal of the Science of Food and Agriculture 26, 1399–1411.

Duodu, K.G., Nunes, A., Delgadillo, I., Parker, M.L., Mills, E.N.C., Belton, P.S., Taylor, J.R.N., 2002. The effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. Journal of Cereal Science 35, 161–175.

Duodu, K.G., Taylor, J.R.N., Belton, P.S., Hamaker, B.R., 2003. Factors affecting sorghum protein digestibility. Journal of Cereal Science 38, 117–131.

Emmambux, M.N., Taylor, J.R.N., 2003. Sorghum kafirin interaction with various phenolic compounds. Journal of the Science of Food and Agriculture 83, 402–407.

Emmambux, M.N., Stading, M., Taylor, J.R.N., 2004. Sorghum kafirin film property modification with hydrolysable and condensed tannins. Cereal Science 20, 127–135.

Edens, L., Van der Laan, J.M., Craig, H.D., 2005. Mechanisms and prevention by proline-specific proteases. Brauwelt International 3, 157–161.

Evans, D.J., Schüssler, L., Taylor, J.R.N., 1987. Isolation of reduced-soluble protein from sorghum starchy endosperm. Journal of Cereal Science 5, 61–65.

Gallagher, S.R., 1999. Electrophoretic separation of proteins. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.E., Seidman, J.G., Smith, J.A., Strule, K. (Eds.), Current Protocols in Molecular Biology. Wiley, New York, p. 10.2A9.

Gao, C., Taylor, J., Wellner, N., Byaruhanga, Y.B., Parker, M.L., Mills, E.N.C., Belton, P.S., 2005. Effect of preparation conditions on protein secondary structure and biofilm formation of kafirin. Journal of Agricultural and Food Chemistry 53, 306–312.

Hancock, J.D., 2000. Value of sorghum and sorghum co-products in diets for livestock. In: Smith, C.W., Frederiksen, R.A. (Eds.), Sorghum, Origin, History, Technology and Production. Wiley, New York, pp. 731–749.

Hamaker, B.R., Kirleis, A.W., Butler, L.G., Axtell, J.D., Mertz, E.T., 1987. Improving in-vitro protein digestibility of sorghum with reducing agents. Proceedings of the National Academy of Science USA 84, 626–628.

International Organization for Standardization (ISO), 1988. Sorghum: determination of tannin content, ICS:67.060, Food Technology: Cereals. Pulses and Derived Products. International Organization for Standardization, ISO 9648, Paris.

Kim, C.S., Woo, Y., Clore, A.M., Burnett, R.J., Carneiro, N.P., Larkins, B., 2002. Zein protein interactions, rather than the asymmetric distribution of zein mRNAs on endoplasmic reticulum membranes, influence protein body formation in maize endosperm. The Plant Cell 14, 655–672.

Lawton, J.W., 2002. Zein: a history of processing and use. Cereal Chemistry 79, 1–18.

Lacroix, M., Le, T.C., Ouattara, B., Yu, H., Letendre, M., Sabato, S.F., Mateescu, M.A., Patterson, G., 2002. Use of γ-irradiation to produce films from whey, casein and soya proteins: structure and functional characteristics. Radiation Physics and Chemistry 63, 827–832.

Le Tien, C., Letendre, M., Ispas-Szabo, P., Mateescu, M.A., Demas-Patterson, G., Yu, H.L., Lecroix, M., 2000. Development of biodegradable films from whey proteins by cross-linking and entrapment in cellulose. Journal of Agricultural and Food Chemistry 48, 5566–5575.

Nguz, K., Van Gaver, D., Huyghebaert, A., 1998. *In vitro* inhibition of digestive enzymes by sorghum condensed tannins (*Sorghum bicolor* (L Moench)). Science Alimentaris 18, 507–514.

Salunkhe, D.K., Chavan, J.K., Kadam, S.S., 1990. Dietary Tannins: Consequences and Remedies. CRC Press, Inc., Boca Raton, FL.

Sarni-Manchado, P., Cheynier, V., Moutounet, M., 1999. Interactions of grape seed tannins with salivary proteins. Journal of Agricultural and Food Chemistry 47, 42–47.

Serna-Saldivar, S., Rooney, L.W., 1995. Structure and chemistry of sorghum and millets. In: Dendy, D.A.V. (Ed.), Sorghum and Millets: Chemistry and Technology. American Association of Cereal Chemists, St. Paul, MN, pp. 69–124.

Shewry, P.R., 2002. The major seed storage proteins of spelt wheat, sorghum, millets and pseudocereals. In: Belton, P.S., Taylor, J.R.N. (Eds.), Pseudocereals and Less Well-Known Cereals. Springer, Berlin, pp. 1–24.

Shull, J.M., Watterson, J.J., Kirleis, A.W., 1992. Purification and immunocytochemical localization of kafirins in *Sorghum bicolor* (L. Moench) endosperm. Protoplasma 171, 64–74.

Spencer, C.A., Cai, Y., Gaffney, R.M.S.H., Goulding, P.N., Magnolato, D., Lilley, T.H., Haslam, E., 1988. Polyphenol complexation—some thoughts and observations. Phytochemistry 27, 2397–2409.

Swiss Prot, UniProtKB/TrEMBL entry Q6Q299, 2005. 〈http://www.us.expasy.org/uniprot/Q6Q299〉 accessed 26 October 2005.

Taylor, J.R.N., Belton, P.S., 2002. Sorghum. In: Belton, P.S., Taylor, J.R.N. (Eds.), Pseudocereals and Less Well-Known Cereals. Springer, Berlin, pp. 25–38.