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Kafirin Microparticle Encapsulation of Catechin and Sorghum Condensed Tannins

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To exploit the porous nature of previously developed kafirin microparticles, encapsulation of the bioactive polyphenols, catechin and sorghum condensed tannins, was investigated. The antioxidant release profiles of the encapsulated substances were studied under simulated gastric conditions. Kafirin microparticles encapsulating catechin or sorghum condensed tannins were similar in size to control kafirin microparticles (5−6 μm). TEM showed that kafirin microparticles encapsulating catechin had a rough porous surface. Microparticles encapsulating sorghum condensed tannins were irregular in shape, some apparently joined together, with a mixture of rough and smooth surfaces. Over a period of 4 h, catechin and sorghum condensed tannin encapsulated kafirin microparticles showed virtually no protein digestion but released approximately 70 and 50%, respectively, of total antioxidant activity. Thus, the use of kafirin microparticles to encapsulate catechin and sorghum condensed tannins has potential as an effective method of controlled release of dietary antioxidants.

KEYWORDS: Kafirin; microparticles; encapsulation; controlled release; tannins; polyphenols; antioxidants

INTRODUCTION

Microencapsulation using protein microparticles (microspheres) has been investigated for delayed or controlled release of pharmaceuticals. Some success has been reported using zein microspheres for drug delivery (*1*−*5*). However, early work used toxic chemicals such as dimethyl sulfoxide for microsphere preparation (*1*, *2*), and drug encapsulation efficiencies were low.

Extensive homology exists between kafirin and zein (*6*). The two prolamins have similar molecular weights and are structurally related (*7*), but kafirin is more hydrophobic than zein (*8*) and less digestible (*9*). These differences may enable kafirin microparticles to be produced with properties superior to those of zein microparticles. An ethanol-free method of preparing kafirin microparticles has been devised (*10*), which enables the production of porous microparticles with vacuoles. These microparticles have a large internal surface area and thus have potential as encapsulating agents.

Recently, there has been considerable interest in the positive health aspects of sorghum polyphenols because they exhibit considerable antioxidant activity (*11*). When compared to blueberries, they have similar antioxidant levels and higher levels than other cereals and fruits such as strawberry and plums (*12*). Sorghum consumption reduces the risk of some types of cancer in humans, and the high levels of sorghum polyphenols are thought to be responsible (*11*).

The antioxidant activity of polyphenols in the body is dependent on their degree of absorption and metabolism (*13*). Only a fraction of the total amounts of food polyphenols are absorbed in vivo. This is thought to be due to factors such as poor solubility, inefficient permeability, instability due to food storage, first-pass metabolism before reaching the systemic circulation, and gastrointestinal degradation (*14*). To take maximum advantage of the potential therapeutic benefits of antioxidants, novel methods of delivery are required.

The aim of this work was to exploit the interactions between sorghum polyphenols and proteins, such as cross-linking (*15*, *16*) and the resulting decrease in protein digestibility (*17*, *9*) to develop a potential new delivery vehicle for dietary polyphenol antioxidants, by encapsulation within kafirin microparticles. To help achieve this, the release profiles of these antioxidants and the enzymic degradation of the kafirin microparticles were investigated under simulated gastric conditions.

MATERIALS AND METHODS

Materials. A mixture of two condensed tannin-free, tan plant, white sorghum cultivars (*Sorghum bicolor* (L.) Moench.) PANNAR PEX 202 and 206 was used for kafirin extraction (*10*).

Condensed tannin was extracted from a red type III tannin sorghum (ex. Nola GH91) (*15*) and used for encapsulation in kafirin microparticles. The condensed tannin content of the extract was 2730 mg/g (dry weight) (catechin equivalents). Catechin (Sigma, St. Louis, MO) was also used for encapsulation in kafirin microparticles.

Pepsin (EC 3.4.23.1) (Merck, Darmstadt, Germany), trypsin (Sigma), and chymotrypsin (EC 232.671.2) (Sigma) were used for protein digestibility determination.

Preparation of Kafirin Microparticles. Kafirin microparticles were made by phase separation from an organic acid (*15*). Freeze-dried microparticles were prepared by removing the dilute acetic acid in which

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the microparticles were suspended by centrifugation and washing the pellet containing the microparticles with distilled water. This was repeated three times before the resultant pellet was freeze-dried.

Encapsulation of Catechin and Sorghum Condensed Tannins in Kafirin Microparticles. Two phenolic compounds were chosen for encapsulation by kafirin microparticles. They were catechin, which has not been found to complex with kafirin, and an extract of sorghum condensed tannins, which has been found to complex with kafirin (*15*). Kafirin microparticles were used to encapsulate catechin and sorghum condensed tannins at a level of 20% polyphenol in relation to protein. Catechin and sorghum condensed tannin extracts (400 mg) were dissolved separately in 10 mL of 70% aqueous acetone. A portion (3.75 mL) of each polyphenol solution was individually mixed by stirring, with 750 mg of freeze-dried kafirin microparticles. Samples were left for 1 h before drying overnight at ambient temperature (25 °C). The resultant material was ground to a fine powder using a mortar and pestle. Two samples of each polyphenol/kafirin mixture were prepared.

Scanning Electron Microscopy (SEM). Preparations of kafirin microparticles were mounted on a stub with double-sided tape and sputter coated with gold. SEM preparations were viewed with a JEOL JSM-840 SEM (Tokyo, Japan). The size of the microparticles was determined by comparing the microparticle images with that of a scale bar of the same magnification. At least 100 microparticles of each treatment were measured.

Transmission Electron Microscopy (TEM). Preparations of kafirin microparticles were prepared for TEM by fixing in gluteraldehyde in pH 7.4 phosphate buffer before staining with osmium tetraoxide. Samples were dehydrated sequentially in acetone before being infiltrated with Quetol resin and polymerized at 60 °C. Sections were cut and stained with ural acetate and lead citrate. TEM preparations were viewed with a Philips EM301 TEM (Eindhoven, The Netherlands).

Dissolution and Release of Antioxidant Activity by Simulated Digestion. Sodium citrate buffer, 0.1 M (0.75 mL), pH 2.0, was added to approximately 20 mg of accurately weighed kafirin microparticles and to kafirin microparticles encapsulating either catechin or sorghum condensed tannins, followed immediately with 1.0 mL of pH 2.0 citrate buffer containing pepsin (367.5 mg of pepsin/100 mL of buffer) and suspended by swirling. Tubes were incubated at 37 °C in a water bath with vortex mixing every 15 min. Samples were taken at intervals up to 2 h and centrifuged at 7200*g* for 5 min to form a firm pellet. The clear supernatant was removed, retained, and immediately frozen (−20 °C) for determination of antioxidant activity of the kafirin microparticles encapsulating either catechin or sorghum condensed tannins. The pellets were washed with distilled water and centrifuged, and the supernatant was discarded. Residues were dried at 100 °C overnight in a forced draft oven, and protein content was determined.

Additional samples were exposed to a 2 h pepsin digestion and centrifuged, and the supernatant was discarded. Immediately, 0.75 mL of 0.1 M sodium phosphate buffer, pH 7.6, was added, followed by a further 1 mL of 0.1 M sodium phosphate buffer, pH 7.6, containing a mixture of trypsin/chymotrypsin (117 mg of trypsin and 138 mg of chymotrypsin/30 mL of 0.1 M sodium phosphate buffer, pH 7.6). Tubes were incubated as previously at 37 °C in a water bath with vortex mixing every 15 min. Samples were taken at intervals up to 2 h, supernatants were retained for determination of antioxidant activity, and the pellet was used for residual protein determination.

Protein digestibilities were determined at each time interval for the pepsin digestion and the trypsin/chymotrypsin digestion. Samples were assayed in duplicate, and two separate runs were performed, giving four results per sample. Another two sets of the same samples were exposed to 2 h of pepsin and 2 h of pepsin followed by 2 h of trypsin/chymotrypsin digestion. These samples were centrifuged and the pellets frozen prior to preparation for TEM or SDS-PAGE.

Protein Digestibility of Protein Preparations. Total protein and residual protein (N × 6.25) of the kafirin microparticle preparations exposed to simulated digestion were determined by a Dumas combustion method (*18*). 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.

ABTS Antiradical Analysis. Antioxidant activity assays were performed on samples of catechin and sorghum condensed tannins used for encapsulation and the supernatant samples using the 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulphonic acid (ABTS) antiradical method (*19*) with Trolox as a standard. Standard curves were prepared with Trolox dissolved in either 5% methanol in 0.1 M sodium phosphate buffer solution, pH 7.6, or 5% methanol in 0.1 M sodium citrate buffer, pH 2.0.

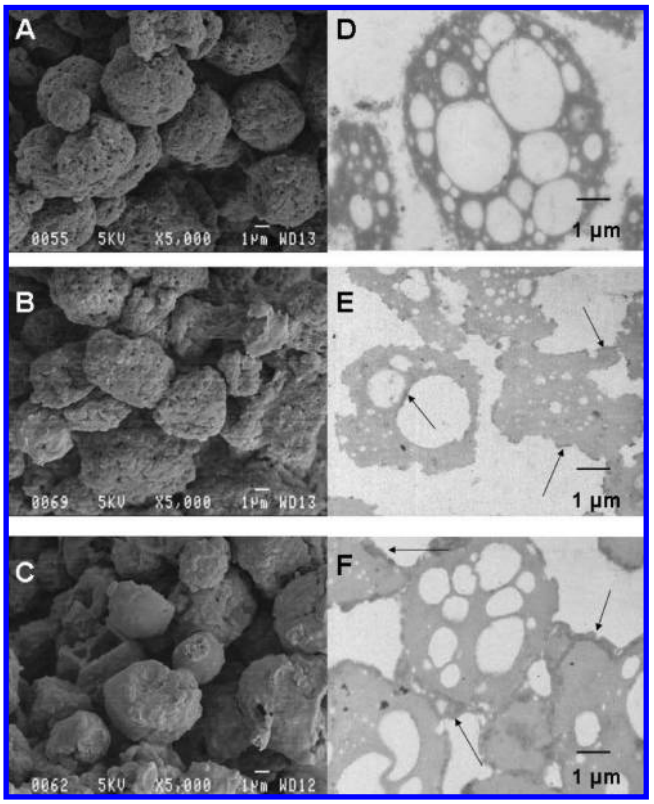

Figure 1. SEM and TEM of kafirin microparticles (**A**, **D**) and kafirin microparticles after encapsulation of catechin (**B**, **E**) and sorghum condensed tannins (**C**, **F**). Arrows indicate probable bound phenolics.

SDS-PAGE. Protein preparations exposed to simulated digestion were characterized by SDS-PAGE on preprepared 4−12% Bis-Tris gradient gels (Invitrogen Life Technologies, Carlsbad, CA) using an X Cell SureLock Mini-Cell electrophoresis unit (Invitrogen Life Technologies) both under reducing and nonreducing conditions. The different protein preparations were loaded to constant protein (≈10 μg) except for the 4 h sample, in each case loaded at ≈10 and ≈15 μg. Molecular weight markers, Invitrogen Mark 12 Unstained standard (Invitrogen Life Technologies), were used. Proteins were stained with Coomassie Brilliant Blue R250 and scanned on a flat bed scanner.

RESULTS AND DISCUSSION

Kafirin microparticles ranged in size from 1 to 10 μm in diameter (**Figure 1**). They were essentially spherical or irregular in shape with a rough porous surface and numerous holes (vacuoles) as shown by SEM (**Figure 1A**) and TEM (**Figure 1D**). Encapsulating catechin or sorghum condensed tannins did not affect microparticle size, the majority having a diameter of 5−6 μm.

Encapsulation efficiency was not quantitatively determined. Osmium staining was used for the TEM to determine the effectiveness of the encapsulation procedure (**Figure 1**). Sorghum tannins are strongly osmiophilic and appear as darkly stained material (*20*). Encapsulation of catechin (**Figure 1B**,**E**) or sorghum condensed tannins (**Figure 1C**,**F**) appeared to have caused a change in the microstructure of kafirin microparticles when compared to the kafirin microparticles not used for encapsulation (**Figure 1A**,**D**). The kafirin microparticles encapsulating catechin appeared to have been eroded at the edges, and the general shape of the microparticles was irregular, with some apparent

aggregation (**Figure 1B**,**E**). These microparticles showed slight darkening around the periphery (**Figure 1E**). This slight darkening is presumably due to the fact that catechin does not complex well with kafirins but seems to show low-level binding at high catechin concentration (*15*). A layer of darkly stained material could be seen clearly around the periphery of the kafirin microparticles encapsulating condensed tannins (**Figure 1F**). The tannins appeared to have linked some of the kafirin microparticles together, forming large irregularly shaped structures. There was also some dark staining material on the inside of the microparticles, mainly on the edge of some of the vacuoles. This indicates that at least some of the sorghum condensed tannins became entrapped within the kafirin microparticles. By implication, because the condensed tannins appear to have been encapsulated at least in part within the kafirin microparticles, then it may be expected that catechin would also have penetrated the interior of the kafirin microparticles.

The catechin and sorghum condensed tannins were found to have antioxidant activities of 15327 and 5366 μmol of Trolox equiv/g, respectively, which are slightly higher than literature values (*21*). However, large variability in antioxidant values is often found (*22*) and is a consequence of the method of analysis and the solvent used to dissolve the polyphenol. Initial antioxidant activity of the encapsulated material was not determined and so is not considered when discussing the following data. At 10 min of pepsin digestion, microparticles encapsulating catechin and microparticles encapsulating sorghum condensed tannins showed a release of antioxidant activity of slightly less than 20% (**Figure 2**). This release of antioxidant activity was probably due to residual, unbound material on the outside or near the surface of the microparticles being washed off with the buffer. This mechanism for the initial release of encapsulated material has been described for zein microparticles (*4*, *5*, *23*). Specific amounts of material released were not mentioned, but it is known that the magnitude of the "burst release" increases with higher loading of encapsulated material (*24*).

Kafirin microparticles encapsulating catechin showed a progressive release of antioxidant activity up until 90 min of pepsin digestion after the initial release (**Figure 2**). At this time, a plateau was reached, with approximately 40% of encapsulated antioxidant activity having been released. On change of pH to 7.6 and enzyme to trypsin and chymotrypsin, a further release of antioxidant activity occurred up to 150 min of digestion (**Figure 2**). Beyond this period a further plateau was reached and very little further antioxidant activity was released. The total antioxidant release from kafirin microparticles encapsulating catechin was slightly less than 70%.

Concerning release of antioxidant activity due to the sorghum condensed tannins (**Figure 2**), after the initial release, there was essentially no further release of antioxidant activity during the period of pepsin digestion. When the pepsin was replaced by trypsin and chymotrypsin in buffer at pH 7.6, there was a further release of antioxidant activity until 150 min; antioxidant activity release then leveled off. The total amount of antioxidant activity released was about 50%.

The additional release of antioxidant activity from kafirin microparticles encapsulating catechin or sorghum condensed tannins that occurred when pepsin was replaced by trypsin and chymotrypsin was probably due to the effect of change in pH from pH 2 to pH 7.6 on kafirin conformation. Changes in pH, ionic strength, and the nature of the ions present are known to affect protein conformation (*25*). Consequently, indirectly this may have resulted in a change in binding properties of the sorghum condensed tannins to the kafirin microparticles, thus allowing the release of further antioxidant activity. There was also

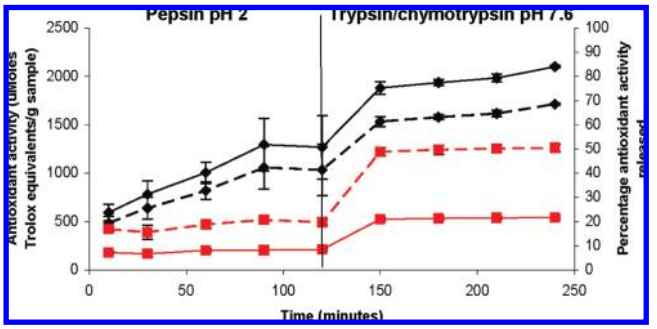

Figure 2. Effect of pepsin digestion followed by trypsin/chymotrysin digestion on antioxidant activity (solid line) and percentage antioxidant released (dotted line) from kafirin microparticles with encapsulated catechin (diamonds) and kafirin microparticles with encapsulated sorghum condensed tannins (squares). Error bars represent ± one standard deviation.

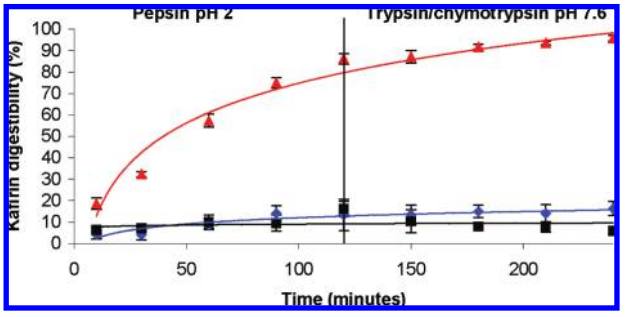

Figure 3. Effect of pepsin digestion followed by trypsin/chymotrysin digestion on kafirin digestibility of kafirin microparticles (triangles), kafirin microparticles with encapsulated catechin (diamonds), and kafirin microparticles with encapsulated sorghum condensed tannins (squares). Error bars represent±one standard deviation.

an increase in antioxidant release for the kafirin microparticles encapsulating catechin. The mechanism of release of active ingredients from microparticles is usually by diffusion but is influenced by the size, shape, and core materials of the microparticles (*26*). Release is also dependent on whether the active ingredient is bound to the core material and whether there is any enzymic degradation of the microparticle (*27*). For kafirin microparticles encapsulating catechin, as described, there was some slight association of catechin to the kafirin (**Figure 1**). The low enzymic digestion of the kafirin (**Figure 3**) was possibly a result of this association. Thus, it is likely that the progressive release of antioxidant activity from the kafirin microparticles encapsulating catechin was primarily by diffusion. The rate of diffusion was initially rapid and then decreased as the distance that the catechin had to diffuse through the kafirin matrix became greater, requiring a longer diffusion time from the interior of the microparticle. It is suggested that the remaining catechin was physically entrapped within the kafirin microparticle and was only released when the conformation of the protein changed with the change in pH at 120 min.

The digestion of control kafirin microparticles was progressive with time. After 120 min of digestion with pepsin, approximately 80% of the kafirin had been digested, rising to approximately 95% after a further 120 min of digestion with trypsin and chymotrypsin (**Figure 3**). This progressive digestion is illustrated by TEM (**Figure 4B**,**C**) and by SDS-PAGE (**Figure 5**). There is little literature on the digestibility of isolated kafirins. The pepsin digestibility of uncooked sorghum protein body enriched samples is about 75% (*28*). The higher protein digestibility of the kafirin

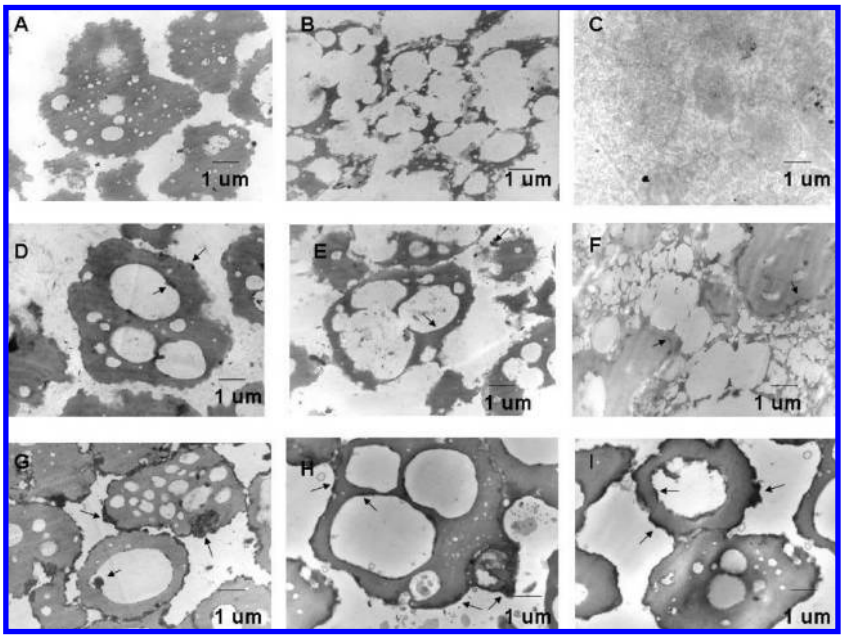

Figure 4. TEM illustrating the effect of pepsin digestion followed by trypsin/chymotrysin digestion on kafirin digestibility of kafirin microparticles (**A**−**C**), kafirin microparticles with encapsulated catechin (**D**−**F**), and kafirin microparticles with encapsulated sorghum condensed tannins (**G**−**I**). Arrows indicate probable bound phenolics.

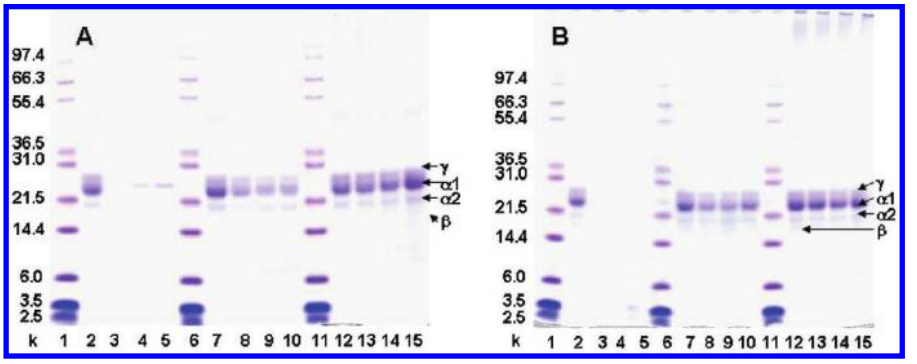

Figure 5. SDS-PAGE of progressive digestion (time 0, 2, 4, and 4 h) of kafirin microparticles (lanes 2−5), kafirin microparticles with encapsulated catechin (lanes 7−10), and kafirin microparticles with encapsulated sorghum condensed tannins (lanes 12−15): (**A**) reducing conditions; (**B**) nonreducing conditions; lanes 1, 6, and 11, MW markers.

microparticles was probably a result of the large surface area of the microparticles available for pepsin attack. Digestion appeared to take place initially both on the outer surface of the microparticles and also from the inside of the vacuoles in the microparticles. This would be expected as the enzyme molecules would be able to penetrate the microparticles via the holes on the surface of the microparticles and through interconnected channels to the interior vacuoles. This pattern of digestion appears to be similar to that of the degradation of sorghum protein bodies during germination (*29*). Protein bodies were degraded by progressive reduction in size from the surface and in some cases appeared to have holes in the central region. As digestion of kafirin microparticles progressed it appeared that some of the vacuoles merged, forming larger structures (**Figure 4B**). Finally, at the end of digestion very little material was left (**Figure 5**) and no specific structure remained (**Figure 4C**).

When the pepsin digestion of the kafirin microparticles encapsulating catechin was measured, there was very little kafirin digested over the 2 h period (**Figure 3**). However, TEM of this material revealed some degradation from the outside of the microparticles (**Figure 4D**,**E**). There also appears to be some breakdown of the internal walls between the vacuoles (**Figure 4E**) when compared to the same microparticles before pepsin digestion (**Figure 4D**). SDS-PAGE after 2 h of pepsin digestion also showed a decrease in intensity of all kafirin bands (**Figure 5**, lanes 8) compared with the starting material (**Figure 5**, lanes 7). There was also very little measurable digestion of the kafirin microparticles encapsulating catechin by the trypsin and chymotrypsin, although, similarly, there was a further decrease in band intensity when the material was subjected to SDS-PAGE (**Figure 5**, lanes 9 and 10). TEM of these microparticles after an additional 2 h of digestion with trypsin and chymotrypsin showed extensive changes of their structure (**Figure 4F**) when compared to the same microparticles after just pepsin digestion (**Figure 4E**). After a further 2 h of digestion with trypsin and chymotrypsin, the structure of the individual microparticles could no longer be seen (**Figure 4F**); rather, the material resembles a continuous matrix with vacuoles embedded within it.

There was essentially no measurable amount of kafirin digested from the kafirin microparticles encapsulating sorghum condensed tannins over the whole period regardless of the enzyme used (**Figure 3**). This is not surprising because sorghum condensed

tannins are known to bind to protein (*15*). The binding of the sorghum condensed tannins to the kafirin may have had the effect of rendering the microparticles indigestible, possibly reducing enzyme accessibility, or the tannin may have bound to the digestion enzymes, causing their inhibition, or possibly both. Sorghum tannins have been shown to associate strongly with sorghum prolamins, and these complexes are found as indigestible residues after pepsin digestion (*30*). These workers also found that purified digestive enzymes (as used in this study) were inhibited by tannin. After 2 h of pepsin digestion, TEM of kafirin microparticle encapsulating sorghum condensed tannin revealed very slight degradation (**Figure 4H**) when compared to control kafirin microparticles after the same period of pepsin digestion (**Figure 4B**). SDS-PAGE showed extensive degradation of the control kafirin microparticles after 2 h of pepsin digestion with very few kafirin bands remaining (**Figure 5**, lanes 3), whereas the band pattern of kafirin microparticle encapsulating sorghum condensed tannin (**Figure 5**, lanes 13) was similar to that of the undigested material (**Figure 5**, lanes 12). After a further 2 h of digestion with trypsin and chymotrypsin, there was still very little evidence of degradation of the kafirin microparticles encapsulating sorghum condensed tannins (**Figure 4I**), whereas by this time there were essentially no control kafirin microparticles remaining (**Figure 4C**). Again, this was confirmed by SDS-PAGE. Lanes 14 and 15 of **Figure 5** show little degradation of the kafirin microparticles encapsulating sorghum condensed tannin after a further 2 h of digestion with trypsin and chymotrypsin, whereas lanes 4 and 5 of **Figure 5** show little residual kafirin.

Despite there being essentially no enzymic digestion of the kafirin microparticles encapsulating polyphenols, the total release of antioxidant activity over an extended period of time was relatively high, being approximately 70% from the kafirin microparticles encapsulating catechin and approximately 50% from the microparticles encapsulating sorghum condensed tannin. In addition, it has been demonstrated that sorghum tannins complexed with protein retained at least 50% of their antioxidant activity (*31*). These workers suggest that this bound material acts as a free radical sink within the gastrointestinal tract, thus sparing other antioxidants. Thus, there is the possibility that an additional 25% of the antioxidant activity of sorghum condensed tannins encapsulated in kafirin microparticles would not be lost but would be available to act as a free radical sink within the gastrointestinal tract.

The findings of this work show that kafirin microparticles have potential for encapsulating phytochemicals such as phenolic antioxidants to give controlled release of the antioxidant activity in a system that simulates digestion.

ABBREVIATIONS USED

SEM, scanning electron microscopy; TEM, transmission electron microscopy; ABTS, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid); SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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