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Formation of kafirin microparticles by phase separation from an organic acid and their characterisation☆

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ABSTRACT

Protein microparticles (microspheres) have numerous food and pharmaceutical applications. However, generally preparation of prolamin protein microparticles involves aqueous ethanol as a solvent. An ethanol-free method of making microparticles from kafirin with a novel structure was devised. Glacial acetic acid or other organic acids were used as kafirin solvent and the microparticles formed by phase separation on addition of water. The kafirin microparticles were characterised by light microscopy, scanning electron microscopy and transmission electron microscopy and their size distribution was measured. The kafirin microparticles prepared by phase separation from organic acid were spherical or irregular shaped, between 1 and 10 μm in diameter, with rough, porous outer surfaces and many internal holes or vacuoles. The holes seem to be the footprint of air bubbles which were entrapped during microparticle preparation. With an increase in the final concentration of acetic acid, the structure of the microparticles changed from porous spheres to an open matrix, with a concomitant change in kafirin secondary structure from α-helical to β-sheet, indicative of protein aggregation. These highly vacuolated and open matrix type microparticles appear to have potential as encapsulating agents and support structures.

1. Introduction

Kafirin, the sorghum prolamin storage protein, by definition aqueous alcohol-soluble, has been found to be readily soluble in glacial acetic acid at ambient temperature (Taylor et al., 2005). Subsequently, it was observed that when water was slowly added to a solution of kafirin in glacial acetic acid, phase separation occurred and the kafirin then precipitated out, as the solvent became more hydrophilic. This phenomenon seems to be similar to the aggregation behavior of zein in aqueous ethanol, observed by Kim and Xu (2008).

When the precipitated kafirin aggregates were observed by light microscopy, they were found to be spherical in nature and similar to zein microspheres, which have been described by various authors (Cook and Shulman, 1998; Demchak and Dybas, 1997; Dong et al., 2004; Liu et al., 2005; Parris et al., 2005; Wang et al., 2005). When phase separation is used for zein microparticle preparation, the zein is generally dissolved in aqueous ethanol. Water or an acid is used as the second solvent. This is essentially the procedure used by Kim and Xu (2008) in their investigation of zein aggregation.

This paper describes the characterisation of kafirin microparticles formed by phase separation from an organic acid.

2. Experimental

2.1. Materials

Grain of a mixture of two condensed tannin-free, tan plant, white sorghum cultivars PANNAR PEX 202 and 206 were used for kafirin extraction, using the method described by Emmambux and Taylor (2003). Decorticated, milled grain, particle size less than 0.8 mm was extracted with 70% (w/w) aqueous ethanol containing 5% sodium hydroxide (w/w) and 3.5% sodium metabisulphite (w/w) at 70 °C for 1 h with vigorous stirring. The extractant was recovered by centrifugation and the ethanol removed by evaporation. Kafirin was precipitated on pH adjustment of the protein suspension to approximately pH 5 and recovered by filtration under vacuum and freeze dried. The kafirin was defatted with hexane at ambient

Abbreviations: ANOVA, analysis of variance; LM, light microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; FTIR, Fourier transform infrared spectroscopy; GMP, glutenin macropolymer.

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temperature and air dried. The protein content of the kafirin was 88% (as is basis, remainder mainly moisture) (N × 6.25) as determined by a Dumas combustion method, American Association of Cereal Chemists (AACC, 2000) Approved Method 46-30.

2.2. Methods

2.2.1. Preparation of kafirin microparticles with acetic acid

Plasticiser (0.66 g of 1:1:1 lactic acid, polyethylene glycol 400, glycerol) equivalent to 40% in relation to protein was mixed with 4.34 g glacial acetic acid (98%) (Merck, Wadeville, South Africa) and added to kafirin (1.8 g, 88% protein, as is basis) with gentle stirring using a magnetic stirrer. The temperature was slowly raised to 30 °C to ensure full solvation of the kafirin. This solution was then allowed to 'rest' for 16 h to give time for any changes in protein structure to equilibrate. After this period, distilled water at ambient temperature was added slowly over a period of 5 min with stirring to a total weight of 80 g. During addition of the water, microparticles formed. The final concentration of protein was 2% (w/w), with an acetic acid concentration of 5.4% (w/w). Freeze dried microparticles were prepared by removing the acid by centrifugation and washing the pellet of microparticles three times with distilled water. The supernatant was removed before freeze drying the resultant pellet.

2.2.2. Preparation of kafirin microparticles with lactic acid or propionic acid

The method was followed as above, substituting lactic acid or propionic acid for glacial acetic acid. When lactic acid was used, all the lactic acid was absorbed by the kafirin. A further 5 g of lactic acid was needed before a solution was formed.

2.2.3. Preparation of kafirin microparticles without plasticiser

Kafirin microparticles were prepared as described above but without the addition of plasticiser.

2.2.4. Preparation of kafirin microparticles with gas saturated or degassed solvents

Glacial acetic acid and distilled water were degassed by boiling for 10 min and then sealed in gas tight bottles to cool. Glacial acetic acid and distilled water were saturated with gas by bubbling air through the solvents for 1 h and then sealed in gas tight bottles prior to use. Kafirin microparticles were then prepared by the method described above without the addition of a plasticiser, either using the degassed or gas saturated solvents.

2.2.5. Preparation of kafirin microparticles with aqueous ethanol

Kafirin (1.26 g, 88% protein) was dissolved in 70% (w/w) aqueous ethanol (15 ml) at 70 °C. The solution was allowed to 'rest' for 16 h. On cooling, the kafirin precipitated. The suspension was reheated to dissolve the kafirin before distilled water was added slowly over a period of 5 min with stirring to a total weight of 55 g, to give a 2% concentration of protein. As with the acetic acid method, the microparticles formed on addition of water.

2.2.6. Effect of shear on microparticle formation

Microparticles were prepared in acetic acid or aqueous ethanol as described above but water was added whilst samples were mixed with an Ultra Turrax (Janke and Kunkel, IKA Labortechnik, Staufen, Germany) at 13,500 rpm for 2 min.

2.2.7. Size, shape and size distribution of kafirin microparticles

Suspensions of kafirin microparticles, 2% protein (w/w), in different acid concentrations were prepared by centrifuging aliquots of microparticles suspended in 5.4% acetic acid at 3880 g for 5 min, decanting off the supernatant and replacing it with an equivalent weight of higher acetic acid concentration (10.8%, 21.6%, 30%, 40%) containing placticiser. Samples were mixed and left overnight before viewing and photographing under phase contrast conditions using a Nikon Optiphot light microscope (Kanagawa, Japan). Size of the microparticles was determined by comparing the micoparticle images with that of a scale bar of the same magnification. At least 150 microparticles of each treatment were measured. These microparticles were compared with aqueous ethanol prepared kafirin microparticles.

2.2.8. Electron microscopy of microparticles

Wet preparations of microparticles were prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) by removing the liquid fraction and fixing in glutaraldehyde in pH 7.4 phosphate buffer before staining with osmium tetraoxide. Samples were dehydrated sequentially in acetone. TEM samples were infiltrated with Quetol resin and polymerized at 60 °C. Sections were cut and stained with ural acetate and lead citrate. SEM samples were subjected to critical point drying before mounting on a stub and sputter coated with gold. Freeze dried microparticles were mounted on a stub with double sided tape and sputter coated with gold. TEM preparations were viewed with Philips EM301 Transmission Electron Microscope (Eindhoven, Netherlands). SEM preparations were viewed with a Joel JSM-840 Scanning Electron Microscope (Tokyo, Japan).

2.2.9. SDS-PAGE

Kafirin microparticles were characterised by SDS-PAGE on a 4–18% acrylamide gradient both under reducing and non-reducing conditions, as described by Taylor et al. (2007).

2.2.10. FTIR

Samples were scanned using a Perkin Elmer Spectrum GX FTIR system (Waltham, MA, USA) using 32 scans, 8 cm^{-1} band and a sampling interval of 1 cm. Attenuated Total Reflectance mode used a zinc selenide crystal. The FTIR spectra were Fourier-deconvoluted with a resolution enhancement factor of 2 and a bandwidth of 12 $cm^{-1.}$

3. Results and discussion

3.1. Morphology of kafirin microparticles

Microparticles resulting from the glacial acetic acid method described above had different sizes and shapes to those made by an aqueous ethanol method similar to that described by Parris et al. (2005) (Fig. 1a, c SEM outer surface, Fig. 1e, g TEM inner surface). Generally the kafirin microparticles made using glacial acetic acid as kafirin solvent, were spherical or irregular in shape with a rough porous surface and numerous internal holes or vacuoles, as shown by SEM (Fig. 1a, outer surface) and TEM (Fig. 1e, internal structure) respectively. The large number of holes or vacuoles resulted in the formation of microparticles with a very large total surface area. Aqueous ethanol prepared microparticles were mainly small, smooth spheres (Fig. 1c) with no or very few internal holes (Fig. 1g). The larger of the aqueous ethanol prepared microparticles did have relatively more internal holes (Fig. 1g).

It was thought possible that the composition of the protein, the method of protein preparation or the difference in solvents could be responsible for the differences in microparticle morphology. Gao et al. (2005) found that the presence of sodium hydroxide as part of the kafirin extractant improved the yield of kafirin extracted by deamidation of the glutamine residues, thus improving the kafirin solubility. However, these workers showed that the effect of drying