EXECUTIVE SUMMARY

PROJECT TITLE

Analysis of zein from South African maize of variable endosperm texture

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Objective

The objective of this study was to characterize zein from various South African maize hybrids and their respective parent lines. This progress report covers the optimisation of a procedure to characterise zein using matrix assisted laser ionization desorption time-of-flight mass spectrometry (MALDI-TOF MS).

Executive summary

Maize is an important crop for both human and animal consumption. Maize kernel texture (kernel hardness) is an important quality trait for many sectors in the South African maize industry, where a harder texture is desired. Both total protein content and the main storage proteins, zein, have been associated with kernel texture.

Zein is the prolamin (alcohol soluble protein) of maize. It is the major storage protein and constitutes up to 70% of the total protein in conventional maize varieties and is located in protein bodies in the endosperm (Lending & Larkins, 1989; Prasanna *et al.*, 2001). Zein has been divided into four major classes, namely α-, β-, γ-, (Esen, 1987) and δ-zein (Kirihara *et al.*, 1988; Woo *et al.*, 2001); each differing in abundance, molecular weights, solubility and pI values. Zein is important for its commercial application as an industrial polymer (Shukla & Cheryan, 2001), impact on the nutritional protein quality of maize (Gibbon & Larkins, 2005) and, together with total protein content, for their relationship to kernel texture (Dombrink-Kurtzman & Beitz, 1993; Mestres & Matencio, 1996; Blandino *et al.*, 2010). The latter formed the main focus of this study.

A procedure for matrix assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS) was optimised to characterise zein from three field replicates of ten white maize hybrids, grown at three localities, and their respective inbred parent lines. Zein from hybrids was quantified using reverse-phase high performance liquid chromatography (RP-HPLC). Kernel texture of hybrids was assessed with a particle size index (PSI) procedure. Total protein content was also determined.

The zein profiles of these hybrids were evaluated to determine the difference in zein expression. Results of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) indicate α-zeins comprise two (19 kDa and 22 kDa) γ-zeins comprise three (27 kDa, 16 kDa and 50 kDa) and δ-zein comprise two (10 kDa and 18 kDa) sub-classes (Woo *et al.*, 2001). The subclasses of α-zeins can further be divided into a family of homologous proteins with similar molecular weights. SDS-PAGE has classically been used to characterise zein proteins. This technique however, cannot distinguish between proteins with similar molecular weights. MALDI-TOF MS analysis of zein has been reported to provide better detection of zein classes (Adams *et al.*, 2004). The zein extraction and matrix preparation procedure for MALDI-TOF MS analysis was optimised in this research study.

Optimisation was predominantly based on the behaviour of the water soluble 27 kDa γ-zein with respect to two matrices [2-(4-hydroxyphenylazo)benzoic acid (HABA) and α-cyano-4-hydroxy-cinammic acid (CHCA)] and the pH of the matrix solution. Two extraction temperatures (ambient and 60°C) and extraction from defatted (DF) and non-defatted (NDF) maize meal was also investigated. Zein extracted from NDF maize meal at ambient temperature and the use of these matrices in combination dissolved in a 70% acetonitrile (ACN) solution (pH 2.9) gave optimal results in terms of signal-to-noise (S/N) ratio and detection of all major zein classes. The advantage of this procedure is that defatting of the maize meal prior to extraction and an elevated extraction temperature was eliminated. The CHCA matrix is a more water soluble matrix and improved the detection of the 27 kDa γ-zein. Particle size of maize meal did not influence the quality of spectra and maize milled using a 1 mm sieve was sufficient for zein extraction. Using MALDI-TOF MS, seven of the possible nine α-zein peaks (Woo *et al.*, 2001) were observed for the

hybrids and inbred maize lines in this study. Additional peaks corresponding to β-, 27 kDa γ-and δ-zeins were also observed. This could possibly be the result of allelic variation, but needs to be confirmed with detailed genetic studies.

Similarities in spectral profiles were observed between hybrids and their respective parent lines. Zein profiles of hybrids appeared to be a combination of their parent lines. Zein profiles of most hybrids did not differ between localities. Overall, these zein profiles could not have been gained using conventional SDS-PAGE and illustrated the value of MALDI-TOF MS analyses when assessing zein profiles.

Maize hardness is an important quality trait for many sectors in the maize industry. It is a positive trait for dry milling and a negative trait for wet milling. Maize endosperm constitutes up to 80% of the total kernel and consists of two types: vitreous (hard) and floury (soft). In South Africa dry milling is used for production of maize grits, samp, maize meal, and adequate hardness is necessary to obtain optimum yield and milling characteristics. Maize with good milling characteristics have a higher proportion of vitreous endosperm and will give a greater yield of larger maize grits, with a higher economic value. Thus, breeders aim to increase the proportion of the vitreous endosperm in newly released maize hybrids. At present maize kernel texture in breeding lines is assessed by visual inspection; protein and more specifically zein contents are not characteristics considered.

Results presented in this and other studies (Paulis *et al.*, 1993; Pratt *et al.*, 1995; Mestres & Matencio, 1996; Lee *et al.*, 2006; Blandino *et al.*, 2010) indicated total protein and zein proteins (assessed using RP-HPLC) play a role in kernel texture, and should be considered when evaluating kernel texture. In this study a two sieve method was used to assess kernel texture. Two sieves (150 µm placed on top of a 75 µm) fitted with a receiving pan were used to separate maize meal fractions after milling through a 1 mm sieve. Three PSI values were calculated, PSI-1 (fraction retained in 150 µm sieve), 2 (fraction retained in 75 µm sieve), and 3 (fraction obtained in receiving pan). A higher PSI-1 value indicated a harder kernel texture and a higher PSI-2 and PSI-3 the opposite.

The total protein content differed significantly ($P<0.01$) between localities, implying environmental factors and/or genotypes played a role. Kernel texture also varied between localities, with a higher protein content correlating positively with a harder kernel texture. Although discrepancies existed between PSI values and protein content, visual inspection indicated kernels with a higher proportion of floury endosperm tended to have a lower degree of hardness (lower PSI-1 and higher PSI-2 and -3) and protein content.

Similar to MALDI-TOF MS zein profiles, RP-HPLC chromatographic zein profiles of most hybrids did not differ between localities, implying the environment did not have a big impact on zein expression patterns. This was consistent with a previous study reporting the environment had minimal influence on chromatographic profiles (Paulis, 1990). Total area (zein) correlated positively with protein content. As mentioned protein content differed significantly ($P<0.01$) between localities and, thus, total zein content was subsequently influenced. This implied environmental factors most likely influenced the amount of zein proteins expressed. Exposure of maize plants to elevated temperatures (Monjardino *et al.*, 2005) and variation in nitrogen fertilizer rates (Tsai *et al.*, 1980) have been shown to influence zein content.

Correlations and principal component analysis (PCA) indicated if β- and γ-zeins (zein-2) were collectively expressed as percentage area of total RP-HPLC peak area, negative correlations with PSI-1 was observed. When expressed as integrated area (proportional to amount of proteins) given as arbitrary units (AU), a positive correlation was obtained. Therefore, the amount of these proteins present, influences kernel texture. A similar trend was observed for the individual β- and γ-zeins peaks. This was in agreement with results obtained from scanning electron microscopy

(SEM) micrographs. The floury endosperm of the harder hybrid appeared to have more protein bodies than that of a softer hybrid. It has been suggested the protein bodies of floury endosperm are less mature; the floury endosperm contains smaller protein bodies due to the presence of less α-zein and more β- and γ-zeins (Lending & Larkins, 1989). This can possibly explain the positive correlations obtained between β- and γ-zeins when expressed as amount present. Total zein and most individual α-zeins sub-classes contents, correlated positively, whereas certain α-zein sub-classes correlated negatively with a harder kernel texture.

Two hybrids that differed significantly ($P<0.01$) in kernel texture, one having the highest PSI-1 value (hard kernel) and the other the lowest (soft kernel), were subjected to scanning electron microscopy. The vitreous endosperm between the two hybrids was similar; starch granules and proteins were densely packed forming a smooth surface. The starch granules in the floury endosperm of the softest hybrid were spherical and loosely packed whereas starch granules appeared to be more densely packed in the hardest hybrid. Therefore, indicating starch also plays an important role in kernel texture. Total starch content has been negatively linked to a harder kernel texture (assessed with various hardness measurements) whereas protein content was positively linked (Blandino *et al.*, 2010). This was expected; if starch content increases the protein content is diluted. A higher amylose:amylopectin ratio correlated positively with harder kernel texture and total protein content. Amylose has also previously been positively linked to a harder kernel texture due to its less crystalline structure, resulting in a higher degree of compressibility and subsequent denser packing of starch granules (Dombrink-Kurtzman & Knutson, 1997). Analyses of these starch types should, therefore, be considered in future studies.

Correlations obtained in this study between protein content, PSI values and RP-HPLC zein data, were not strong and/or significant. The explained variation by the first two principal components was also not high. Stronger positive correlations for total protein content with other hardness measurements have been reported (Blandino *et al.*, 2010). Overall weak correlations ($r<0.5$) have been reported between zein contents (assessed with RP-HPLC) and degree of hardness (Paulis *et al.*, 1993; Pratt *et al.*, 1995; Mestres & Matencio, 1996). In these studies 70 % ethanol was also used as an extraction solvent. It is possible a higher alcohol (e.g. 2-propanol) can result in a more quantitative extraction of these proteins. Thus, higher alcohols should also be considered in future studies. More kernel texture assessment techniques should also be included to provide a better understanding of the relationship between protein content, zein content and kernel texture. Hardness assessment techniques should resemble parameters in industrial milling processes, such as milling time, milling energy, total grit yield and coarse-to-fine ratio (Blandino *et al.*, 2010). In addition, maize constituents and subsequently, kernel texture are influenced by the environment, genotype, maturity level of hybrid and duration of grain fill period. All these parameters should, thus, be considered when evaluating kernel texture in future studies to obtain a better understanding of maize hardness to, subsequently, breed suitable varieties.

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