Reprinted from
South African Journal of Science *Vol. 79* *May 1983*

Electrophoretic Analysis of Reduced Protein Fractions from a New South African High-lysine (Opaque-2) Hybrid and Three Other Opaque-like Maize Types

Maize (*Zea mays* L.) continues to play a significant role as a source of human dietary protein in South Africa, as well as in a number of developing countries in Africa, parts of India and Central and South America. Discovery[1] of the effectiveness of the opaque-2 gene in increasing the lysine and tryptophan concentrations of maize endosperm protein with a resultant marked improvement in protein quality,[2-4] prompted the development of a locally adapted high-lysine hybrid,[5,6] recently released as the first South African white-kernelled high-lysine (opaque-2) hybrid, HL-1. Apart from these beneficial effects on the amino acid composition of maize endosperm, the opaque-2 gene also influences the hardness and outward appearance of hybrid kernels, decreased transluscence or opaqueness providing an easily recognisable indicator for the presence of the gene. Opaqueness of kernel may, however, be caused by other genetic and even environmental factors, particularly drought and frost. The present study was undertaken in order to ascertain whether SDS-polyacrylamide gel electrophoresis of the reduced polypeptides of the major endosperm protein fractions would, on the one hand, provide a suitable tool for identification of opaque-2 hybrids or, on the other, indicate change in the polypeptide make-up of a specific protein fraction as a common factor in opaqueness of kernel irrespective of primary cause. Molecular weight distributions of the reduced polypeptides were determined in order to facilitate comparison with similar studies reported by other workers,[7-13] particularly Misra *et al.*[14] and Paulis *et al.*[15]

Materials and methods

Samples of drought-damaged white dent, a high-lysine hybrid, a waxy cultivar and a floury type maize referred to as '*broodmielies*' (bread maize) were all supplied by the Maize Board, Pretoria. Normal kernelled white dent maize was obtained from a retail supplier as a typical sample of white maize available on the open market. Whole kernels were ground to a fine powder in a Bleuler vibrating ball mill and defatted by stirring for 1 h in petroleum ether (b.p. 40 – 60°) at 4°C. The procedure of Landry and Moureaux (Sequence D)[16] as modified by Soave *et al.*[17] was used to prepare zein and glutelin fractions, corresponding to Landry-Moureaux fractions II to V, from the defatted whole kernel meals. Solvents and extraction conditions are summarized in Table 1. The zein and zein-like mercaptoethanol-alcohol soluble fractions (LM-II and III) were dialysed against 0.05% glycine, the precipitated protein recovered by centrifugation and freeze-dried. The proteins in fractions LM-IV and V were precipitated by dialysis of the respective extracts against water and freeze-dried.

Protein fractions (5 mg) were dissolved with boiling (3 – 5 min) in 5 ml of sample buffer (0.1 M phosphate, pH 7, containing 1 g of SDS and 1 g of mercaptoethanol per 100 cm^3). SDS electrophoresis was carried out according to the usual procedures[18,19] at pH 7 (0.1 M phosphate buffer) in 10% acrylamide-0.27% *N,N'* methylene-bis-acrylamide gel cylinders (100 × 5 ml) containing 1% sodium dodecyl sulphate (SDS), using bromophenol blue as tracking dye. SDS molecular weight markers were obtained from British Drug Houses. After electrophoresis (8 mA per tube), the gels were stained with Coomassie brilliant blue in an aqueous 10% isopropanol-10% acetic acid (volume ratio) solution and destained in a 10% isopropanol-10% acetic acid solution. Gels were photographed with back lighting.

Results and discussion

To facilitate comparison of the more complex electrophoregrams, mean calculated molecular weights together with the range of values obtained with the five types of maize are presented in tabular form. The selected ranges are consistent with an accuracy of ± 5% for molecular weights determined by SDS-PAGE,[20] although for low molecular weights (below 20 000) the method is considered to be accurate only within 18%.[21] Relative staining intensities of individual polypeptide bands are also indicated in the tables.

Fraction LM-II. This fraction has been called zein,[15,16] true zein[14] or zein-1.[22] The electrophoregrams obtained with the five maize types (Fig. 1) show the two major reduced polypeptides characteristic of zeins separated on 10% gels and designated Z1 and Z2,[12] zeins A and B,[9] or α- and β-zeins.[7] Consistent with the findings of others,[12,14] the slower moving polypeptide from the opaque-2 hybrid stained considerably more faintly and gave a much more diffuse band than the corresponding peptide from the other four maize types, indicating that synthesis of this particular polypeptide chain is especially sensitive to the introduction of the O_2-gene.

Literature values for the two reduced polypeptides fall into two distinct sets, namely 25 and 22 kd and 22 and 19 kd, respectively (Table 2). The mean molecular weights obtained in the present study, 24.8 (range: 24.4 – 25.0) and 22.3 (range: 21.9 – 22.5) kilodaltons, conform to the higher literature values. Confirmation of these values was obtained in a re-analysis of the maize samples using a 70% ethanol-0.5% sodium acetate solution in place of 70% isopropanol-0.5% sodium acetate as zein extractant, which gave respective mean molecular weights of 25.2 (range: 24.5 – 25.8) and 22.2 (21.9 – 22.8) kilodaltons.

The faint but distinct polypeptide band (molecular weight 16.9

Table 1. Procedure employed in the sequential extraction of proteins from defatted whole maize meals (2-ME = 2-mercaptoethanol; SDS = sodium dodecyl sulphate).[17]

Fraction[a]	Solvent	Temp (°C)	Agitation time[b] (h)	Solvents used by Landry-Moureaux[16]
LM-1	0.5 M-NaCl (not examined further)	4	1	
LM-II	Isopropanol, 70% + Na acetate, 0.5%	60 20	1 2	Isopropanol, 55% in absence of salts
LM-III	Isopropanol, 70% + 2-ME, 0.6%	20	1	Isopropanol, 55% + 2-ME, 0.6%
LM-IV	Na bicarbonate buffer, pH 10 + 2-ME, 0.6%	20	1	Borate buffer, pH 10 + 2-ME, 0.6%
LM-V	As for LM-IV + SDS, 0.5%	20	1	Borate buffer, pH 10 + 2-ME, 0.6% + SDS, 0.5%

[a]Fractions are designated LM-1, etc. to indicate their equivalence to those obtained by the Landry-Moureaux procedure (sequence D).[16] The solvents used in the latter procedure are given in the last column.
[b]Each extraction was repeated twice.

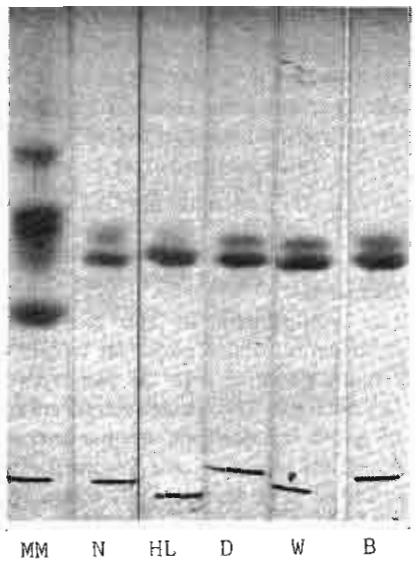

Fig. 1. SDS-polyacrylamide gel (10%) electrophoresis at pH 7 of reduced polypeptides in zein (LM-II; 40 μg) extracted from five types of maize. MM = SDS molecular markers (British Drug Houses). N = normal white dent; HL = high-lysine; D = drought damaged; W = waxy; B = bread (a floury type) maize.

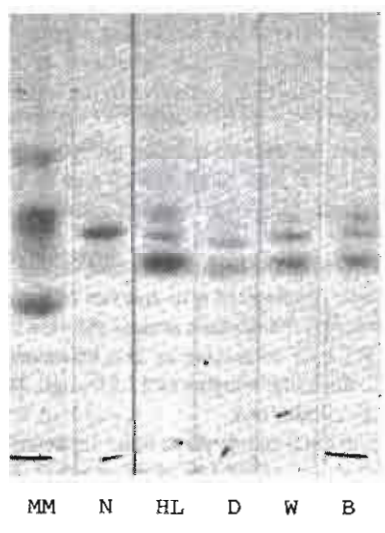

Fig. 2. SDS-polyacrylamide gel (10%) electrophoresis at pH 7 of reduced polypeptides in the mercaptoethanol-alcohol soluble fraction (LM-III) (40 μg) from five types of maize. N = normal white dent; HL = high-lysine (opaque-2); D = drought-damaged; W = waxy; B = bread (floury type) maize. See text for experimental details. MM = SDS molecular weight markers.

kd) discernible in the electrophoregram for the zein from normal maize (Fig. 1) is of questionable significance, as this band was not seen in any of the other gels, was not obtained when normal maize was re-analysed (see above), nor has such a band been reported by other workers using these techniques.

Fraction LM-III. Some confusion exists regarding the identity of this mercaptoethanol-alcohol soluble fraction, which has been variously designated zein-like,[14] zein-2,[22] alcohol soluble reduced glutelins[15] or alcohol soluble glutelins (G_1).[16] The electrophoregrams and molecular weight distributions of the reduced polypeptides are given respectively in Fig. 2 and Table 3. With the exception of the preparation from normal white dent maize, this fraction shows a characteristic 3-banded pattern in the medium molecular weight range. The mean calculated molecular weights, 25.6, 22.7 and 19.1 kd, correspond with molecular weights of 26.0, 23.0 and 18.0 kd reported by Misra *et al.*,[14] who similarly found that these bands were especially well-defined in the case of an opaque-2 hybrid. Surprisingly, the preparation from normal maize examined here shows only one well-developed band (23.1 kd) with a wide diffuse band of mean molecular weight 20.2 kd and no trace of the 25.6 kd polypeptide. Inspection of the electrophoregrams recorded by Misra *et al.*[14] shows that, whereas a clear 3-banded pattern was obtained for one of the normal maize lines (W22), the other (Oh 43) shows only a 2-banded pattern. It appears therefore that there may be variations between normal maize lines with respect to the major reduced polypeptides in this protein fraction.

The strong band at 14.0 kd observed by Misra *et al.*[14] in their opaque-2 mutant was not evident in the gels for the high-lysine (opaque-2) hybrid of the present study, although all the gels show a very faint band at 13.4 kd. On the other hand, the opaque-2 hybrid yielded three definite though faint bands at 35.9, 43.0 and 49.5 kd. The waxy maize showed similar but much fainter bands, while the floury-type maize showed very faint bands at the two higher molecular weights. Misra *et al.*[14] also recorded similar reduced polypeptides at 43.3 and 46.1 kd with a third at 61.0 kd, although it is not clear from their data whether these bands were common to all maize types examined.

Paulis *et al.*,[15] who prepared 'alcohol-soluble glutelins' by a procedure similar to that used here and by Misra *et al.*,[41] but carried out electrophoresis in 5% gels at pH 8.9, did not obtain the 3-banded patterns in the 19 to 25 kd range. They did, however, record four polypeptide bands of mean molecular weights 52.6, 43.9, 24.7 and 13.6 kd, corresponding reasonably well with the bands found respectively at 49.5, 43.0, 25.6 and 13.4 kd in the present study. Their gel patterns are substantially different, however, in that the

Table 2. Literature values for the molecular weights of major reduced polypeptides obtained from alcohol-soluble zeins by SDS-PAGE.

	Molecular weight (kd)		
Experimental conditions	Slower band	Faster band	References
Discontinuous system, separating gel : 15%, pH 6.8	21.8	19.0	12
Discontinuous system, separating gel : 20%, pH 8.9	23.0	21.0	13
10% gel, pH ?	22.5	19.0	8
Not given	21.6	19.6	10
10% gel, pH 7.1	25.0	21.8	14
12.5% gel, pH 8.4	24.0	22.0	11
10 – 15 % gradient gel pH ?	23.9	22.8	9
10% gel, pH ?	25.0	21.0	7

Table 3. Molecular weight distribution of reduced polypeptide chains in the mercaptoethanol-alcohol soluble fractions (LM-III) from N = normal white dent; D = drought-damaged; HL = high lysine; W = waxy; B = bread (a floury type) maize. See text for experimental details. Strongly stained band, + + +, less strongly stained but distinct band, + +; faint band, +; very faint band, (+); diffuse band, diff; gaps indicate no band discernible.

Mol. weight (kd)		Relative band intensity				
Mean	Range	N	D	HL	W	B
49.5	47.2 – 49.6			+	(+)	(+)
43.0	41.5 – 44.9			+	(+)	(+)
35.9	34.3 – 37.5			+	(+)	+
25.6	24.6 – 26.2		+ +	+ +	+ +	+ +
22.7	21.7 – 23.3	+ + +	+ + +	+ + +	+ + +	+ + +
19.1	17.5 – 20.2	diff	+ + +	+ + +	+ + +	+ + +
13.4	12.6 – 13.7	(+)	(+)	(+)	(+)	(+)

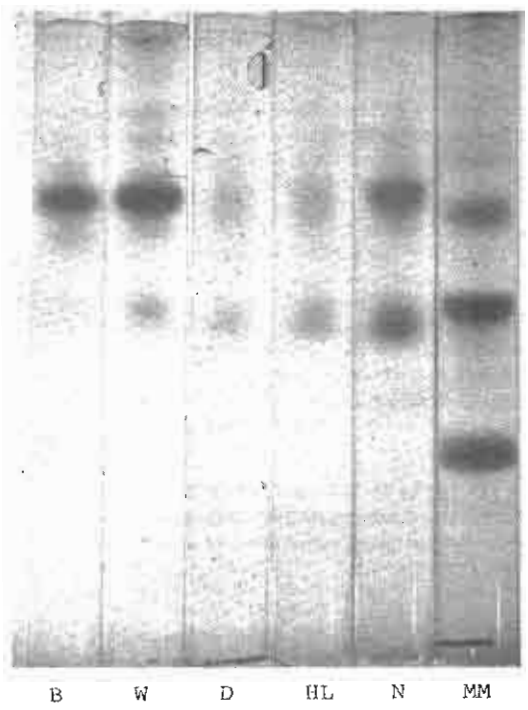

Fig. 3. SDS-polyacrylamide gel (10%) electrophoresis at pH 7 of reduced polypeptides in mercaptoethanol-pH 10 soluble glutelins (LM-IV) from B = bread (a floury type); W = waxy; D = drought-damaged; HL = high-lysine (opaque-2); N = normal white dent maize. See text for experimental details. MM = SDS molecular weight markers.

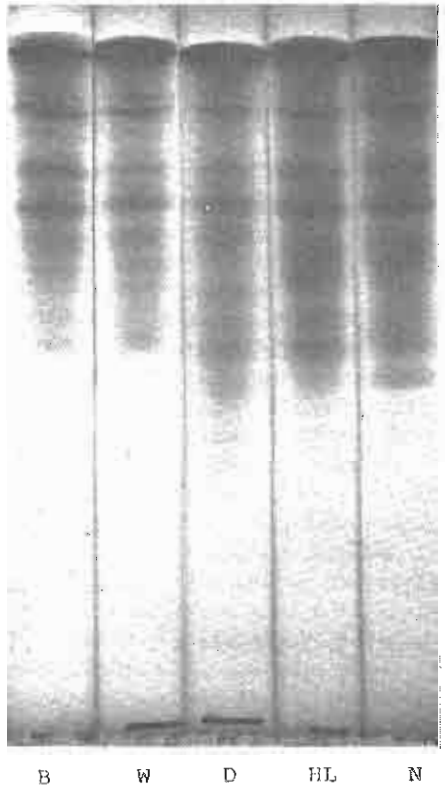

Fig. 4. SDS-polyacrylamide gel (10%) electrophoresis at pH 7 of reduced polypeptides in mercaptoethanol, pH 10, detergent-soluble glutelins (LM-V; 100 μg) from B = bread (a floury type); W = waxy; D = drought-damaged; HL = high-lysine (opaque-2); N = normal white dent maize See text for experimental details. The relevant SDS molecular weight markers are shown in Fig. 3.

band at 43.9 kd is the most intensely stained, while there are no polypeptides at 26 and 23 kd, the dominant bands in the 10% gels obtained here and by Misra *et al.*[14]

Fraction LM-IV. This fraction, designated saline-soluble glutelins (G_2) by Landry and Moureaux[16] and referred to as glutelin-like by Misra *et al.*,[14] may perhaps best be termed mercaptoethanol-pH 10 soluble glutelins. The electrophoregrams obtained with this fraction (Fig. 3 and Table 4) show marked differences between the different maize types as well as some marked discrepancies with respect to the molecular weight distribution reported by Misra *et al.*[14] Whereas the latter state that the darkest staining band from all their samples (which is only poorly visible in the photographs of some of their gels) had an average calculated molecular weight of 25.7 kd, the most intensely stained band in the gels from the normal, waxy and bread (floury) maize studied here had a mean calculated molecular weight of 48.8 kd. The corresponding band for drought-damaged and high-lysine maize is much fainter, staining at the same intensity as the band at 28.3 kd. The 28.3 kd band in the electrophoregrams for normal maize is stained only slightly less intensely than that at 48.8 kd, whereas the equivalent band in waxy and in bread maize is only faintly stained compared with that at 48.8 kd. Other faintly stained bands of higher molecular weight (71.8, 62.3 and 54.9 kd) are seen in the gels for waxy, normal and high-lysine maize. The highest molecular weight species was not evident in the study of Misra *et al.*,[14] who, however, record additional bands at 60.0, 58.0, 38.0 and 11.4 kd. Insufficient data are available to speculate on the significance of the discrepancies noted above, but the available information suggests that this fraction is subject to greater variations between maize types than are the other major protein fractions.

Comparison of these results with those of Paulis *et al.*[15] is made difficult as in the preparation of their 'alcohol-insoluble glutelins' fraction, they combined alkaline conditions with the presence of SDS in the extract, while the pH used, 8.9, was lower than that used here and by Misra *et al.*[14] (pH 10). Their preparation therefore includes proteins from both Landry-Moureaux fractions IV and V. Furthermore, their use of 5% gels resulted in the detection of a number of high molecular weight peptides (90 to 127 kd) not seen here. Of the 17 molecular weight species recorded by these authors, only four, namely those at 54.8, 47.8, 41.4 and 27.7 kd, correspond reasonably closely with those reported here, while five or possibly six others correspond with bands obtained with the detergent-soluble glutelin fraction (LM-V).

Fraction LM-V. The mercaptoethanol-pH 10-detergent-soluble fraction is referred to as glutelins (G_3) or zeanin by Landry and Moureaux[16] and as true glutelins by Misra *et al.*,[14] who reported

Table 4. Molecular weight distribution of reduced polypeptide chains in the mercaptoethanol-pH 10 soluble glutelins (LM-IV). Strongly stained band, + + +; less strongly stained but distinct band, + +; faint band, +; very faint band, (+); gaps indicate no band detected. N = normal white dent; D = drought-damaged; HL = high lysine; W = waxy; and B = bread (floury type) maize.

Mol. weight (kd)		Relative band intensity					Corresponding band observed by
Mean	Range	N	D	HL	W	B	Misra *et al.*[14]*
71.8	71.5 – 71.9	(+)	(+)	(+)	+		—
62.3	61.4 – 62.8	(+)		(+)	+		61.0
54.9	53.3 – 57.3	(+)	+	(+)	+	+	54.4
48.8	47.5 – 49.2	+ + +	+ +	+ +	+ + +	+ + +	47.0
42.8	42.5 – 43.2	(+)	(+)	+	+ +	+ +	40.0
28.3	27.3 – 30.0	+ + +	+ +	+ +	+ +	+	25.7
19.3		(+)	(+)	(+)			19.0
14.6		(+)		(+)			13.4

*These authors report additional faint bands at 60.0, 58.0, 38.0 and 11.4 kd.

that this fraction did not separate clearly on an 8.5% gel. Good resolution was obtained in the present study, however, using a 10% gel but the electrophoregrams (Fig. 4) were complex with at least twelve bands ranging in mean (calculated) molecular weight from 26.1 to 88.8 kd. The gel patterns for the five maize types are remarkably consistent, with dominant bands at 88.0, 64.4 and 55.6 kd, other bands occurring at 88.8, 79.7, 74.1, 59.6, 42.7, 47.2, 35.2, 31.0 and 26.1 kd. The only discrepancies are the lack of the lowest molecular weight polypeptides in the waxy and bread (floury type) maize, the lack of bands at 42.7 and 35.2 kd in drought-damaged maize, and their replacement in this preparation by a band at 35.2 kd, which is also present in the waxy and bread maize preparations. As indicated earlier, five or six of these bands correspond to reduced polypeptides reported by Paulis *et al.*[15] for 'alcohol-insoluble glutelin'.

Conclusion

Although comparison of the results with those of other workers was complicated by differences in procedures followed either in the preparation of fractions or conditions employed for gel electrophoresis, many of the reduced polypeptide bands show good correspondence with those reported by others. Apart from the markedly lower intensity and more diffuse nature of the 25 kd reduced zein polypeptide from the high-lysine hybrid, SDS-PAGE as applied here failed to provide clear-cut distinctions between the maize types investigated. There was also no consistent difference between the four opaque-kernelled maize types as a group and the normal-kernelled maize. The greatest divergence between the five types of maize occurred in Landry-Moureaux Fraction IV preparations, which may therefore prove the most useful fraction for use in genetic specificity studies.

This work was funded by the Department of Agriculture & Fisheries and by the University of Natal. One of us (S. Gorinstein) is indebted to the Department of Agriculture & Fisheries for funding as an Overseas Research Fellow.

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Received 18 November 1982; accepted 16 February 1983.

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