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Characterisation of the Major Prolamins of Tef (*Eragrostis tef*) and Finger Millet (*Eleusine coracana*)

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ABSTRACT

The major prolamins of tef (*Eragrostis tef* (Zucc.) Trotter) and finger millet (*Eleusine coracana* L. Gaertn subsp. *coracana*) were purified and characterised by SDS–PAGE, amino acid analysis and N-terminal amino acid sequencing. These studies indicate that the major prolamins of tef and finger millet are similar to the α-prolamins of the Panicoideae (maize, sorghum and *Coix*), although they are classified in a separate sub-family of the Poaceae, the Chloridoideae.

Keywords: tef; millet, prolamin, N-terminal sequences.

INTRODUCTION

Tef (*Eragrostis tef* (Zucc.) Trotter) and finger millet (*Eleusine coracana*) are two minor cereals, both with their origins in Ethiopia and the Sudan[1,2]. Finger millet has been identified from Neolithic sites in Ethiopia[3] and the Sudan[4], both sites dating to about 3000BC. As such, it may be the oldest indigenous domesticated tropical cereal in Africa. Finger millet was introduced into India during the first millenium BC, being found at a Neolithic site in Mysore, dating to about 1600BC[5]. India then became a second centre for the evolution of the crop. Tef is endemic to the highlands of Ethiopia, cultivation was established in prehistoric times, but exact details are unclear[6]; tef seed and straw have been recognised in Neolithic sites dating to about 2600BC[2]. Unlike finger millet, tef did not leave Ethiopia until the end of the nineteenth century and then mainly as a fodder crop for livestock.

Millet is the common name for a number of small-seeded annual grasses, most of which are adapted to hot, dry climates. All millets, apart from finger millet, are classified in the Paniceae tribe of the Panicoideae, on the basis of morphological characteristics[7]. The major cereals, maize and sorghum, are also classified in the Panicoideae, but in a separate tribe, the Andropogoneae, along with a minor cereal *Coix*[8,9]. Finger millet and tef are classified into the Chlorodoideae sub-family. Other major cereals are placed in different sub-families: the Festucoideae (barley, wheat, rye and oats) and Oryzoideae (rice).

Tef is the staple cereal of Ethiopia, the only country where the grain is used for human consumption. The seeds are small, $\leq 0{\cdot}002$ g, and the flour is used to make several types of flat bread, the most popular being a fermented-circular soft bread

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(injera) which forms the traditional basic diet. The grain can be stored for long periods without insect pest damage, so can be used as a store against times of famine. The straw is used as animal feed and as building material, so that the whole crop is utilised. Tef covers a wide geographical and climate range from 300 m to elevations in excess of 2500 m in both high rainfall areas and in semi-arid regions, covering about 30% of the total cultivated area in Ethiopia. Due to its adaptability it has also been introduced as a forage crop in India, South Africa and Australia.

Finger millet (also known as African millet, koracan or coracan and, in India, ragi) is a staple food in semi-arid regions of Africa and India. The grains are small, 1–2 mm in diameter, and are ground into flour which is made into a thick porridge and also malted to make beer. Like tef, it can be stored for long periods of time without deterioration or insect damage.

In this paper, we present the first detailed characterisation of the prolamins of tef and finger millet, and compare them with the prolamins of other cereals.

EXPERIMENTAL

Protein extraction

Tef and finger millet seed was obtained from Professor E. Bekele, Addis Ababa University, Ethiopia. Total proteins were extracted by grinding seed (50 mg) with 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0·0625 M Tris-HCl, pH 6·8 (1 ml) followed by boiling for 5 min.

Prolamins were extracted from ball-milled seed (10 g), which was defatted with chloroform (2×100 ml) and air-dried. Albumins and globulins were extracted by stirring with 1 M NaCl (2×100 ml) for 1 h and centrifuged (10 000 ***g*** for 15 min), the supernatant solutions were dialysed and freeze-dried. The pellet was washed with water and prolamins extracted with 70% (v/v) aqueous ethanol (2×100 ml for 1 h each), followed by 50% (v/v) aqueous propan-1-ol, 2% (v/v) acetic acid and 2% (v/v) 2-mercaptoethanol (100 ml for 1 h). The respective supernatants were dialysed in a low M_r cutoff membrane (Spectra/Por 3, Pierce and Warriner) and freeze-dried.

Proteins were analysed on 12·5% or 15% (w/v) acrylamide SDS–PAGE gels, based on the system of Laemmli[10].

N-terminal sequencing

Sequencing was performed on an Applied Biosystems Ltd 477A pulsed liquid phase amino acid sequencer with a 120A on-line phenylthiohydantoin (PTH) amino acid analyser.

HPLC analysis

Prior to HPLC analysis prolamin fractions were reduced and alkylated with 4-vinylpyridine[11]. Proteins were separated on a Vydac C_{18} column (218TP1010, 25×1 cm) column, using a linear gradient of 70% buffer B to 100% buffer B over 30 min (buffer A: $H_2O + 0{\cdot}07\%$ (v/v) TFA; buffer B: acetonitrile + 0·05% (v/v) TFA). The flow rate was 2·5 ml/min and the detection wavelength, 225 nm. For analytical HPLC, an identical gradient system was run with a flow rate of 1·0 ml/min, using a Vydac C_{18} column (218TP54, $25 \times 0{\cdot}46$ cm).

Amino acid analyses

Prior to amino acid analysis, prolamins were reduced and alkylated with 4-vinylpyridine to modify cysteine residues, which were detected as S-(4-pyridylthyl)cysteine[11]. Colour correction factors were determined from amino acid standards and acid hydrolysis factors (to compensate for labile amino acid residues) were calculated from a bovine serum albumin (BSA) protein digest. Amino acid analyses were performed in duplicate. Protein samples were hydrolysed in the vapour phase of constant boiling (6 M) HCl (Pierce & Warriner, Sequanal Grade) containing 1% (w/v) phenol in a sealed, heated container under pressure for 1 h. The hydrolysates were reacted with phenylisothiocyanate (PITC) to give phenylthiocarbamyl derivatives which were separated by reverse phase HPLC (RP–HPLC). The results are the means of the duplicate hydrolyses and are expressed as mol%.

RESULTS AND DISCUSSION

Figure 1 shows the SDS–PAGE patterns of the proteins obtained by the sequential extraction of tef and finger millet meals. The albumin and globulin fractions contained many components with a wide range of M_rs (Fig. 1, tracks c and g). In both cases, little protein was extracted with 70% (v/v) aqueous ethanol, the bulk of the prolamins being extracted

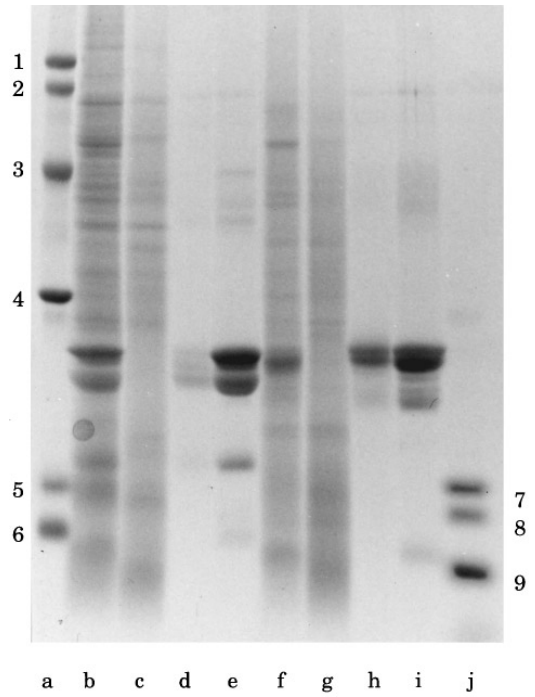

Figure 1 SDS–PAGE of proteins extracted from tef and finger millet. Track a = M_r markers, 1 = 76–78 000, 2 = 66 200, 3 = 42 700, 4 = 30 000, 5 = 17 200 and 6 = 12 300. Tef: track b = total proteins; c = salt extracted proteins; d = 70% ethanol extracted; e = 50% propan-1-ol, 2% acetic acid and 2% 2-mercaptoethanol-extracted. Finger millet: track f = total proteins; g = salt extracted proteins; h = 70% ethanol-extracted proteins; i = 50% propan-1-ol, 2% acetic acid and 2% 2-mercaptoethanol-extracted proteins. Track j = M_r markers 7 = 16 900, 8 = 14 400 and 9 = 8100.

with 50% (v/v) aqueous propan-1-ol in the presence of reducing agent. Two major prolamin bands with M_rs of about 25 000 and 22 500 were present in tef, and three bands with M_rs of about 25 700, 24 500 and 21 900 in finger millet (Fig. 1, tracks e and i). The propan-1-ol extracts also contained minor prolamin bands, with M_rs of about 40 500, 42 500, 48 500 and 60 000 in tef and 45 500 and 60 000 in finger millet (Fig. 1, tracks e and i). The patterns of bands were broadly similar in the two species, with major components in the range M_r 20 000–26 000.

The SDS–PAGE gels indicated that the prolamin fractions of tef and finger millet were less complex than those of wheat, barley and rye, in terms of their apparent molecular size differences, and resembled the pattern found in maize[12]. The SDS–PAGE prolamin patterns were also less complex than those of other members of the Chloridoideae, *Spartina*, *Eleusine indica* and *Cynodon dactylon*[13]. The prolamins were, therefore, fractionated by HPLC on a C_{18} reverse-phase column to isolate individual proteins for further analysis. RP–HPLC separates molecules on the basis of their surface hydrophobicities; six and seven major peaks were resolved in tef and finger millet, respectively (Fig. 2). SDS–PAGE of the peaks separated by semi-preparative HPLC (Fig. 3) showed that peaks 1, 2, 5 and 6 of tef contained single bands, with peaks 1 and 2 corresponding to the major components. In finger millet, only peak 2 was homogeneous, with peak 6 containing the major components. Peaks 2 and 6 of tef and peaks 3 and 6 of finger millet were used to obtain longer N-terminal amino acid sequences and for amino acid analysis.

The amino acid compositions of all the purified fractions were characterised by high contents of glutamine + glutamate (19–32 mol%), proline (9–14 mol%), valine, alanine, leucine, isoleucine and phenylalanine, with arginine, lysine and histidine being present in much lower amounts (Table I). The compositions of the tef and finger millet prolamins showed a high degree of similarity to each other and to the mean values calculated for total prolamins from a number of different finger millet cultivars (Table I). The compositions were unlike those of wheat, barley or rye prolamins, having higher levels of alanine, leucine and isoleucine more similar to the α-prolamins of maize, sorghum and *Coix* in the sub-family Panicoideae.

Table II shows N-terminal sequences of the HPLC-purified tef and finger millet prolamins. The tef proteins gave single dominant sequences, while those for finger millet were much more heterogeneous even after re-purification on an analytical HPLC column, indicating a higher degree of complexity than tef prolamins. The sequences were searched against known prolamin sequences using the Swissprot data base, and alignments made on the basis of homologies.

Tef peak 2 showed a high degree of homology with the α-prolamins of the Andropogoneae (maize, sorghum and *Coix*), the highest being with the Z22 zeins and the kafirins of sorghum (Table II). Tef peak 2 had a deletion of 11 residues at the N-terminus compared with maize Z22, one of 12 residues compared with the maize Z19, sorghum and *Coix* sequences, and showed some 30–40% sequence similarity over the first 30 residues with the α-prolamins. Tef peak 2 also showed sequence homology with the N-terminus of a methionine-rich M_r 10 k (10 kD) rice prolamin (Table II)[14]. However, the amino acid composition of the rice prolamin is quite different from that of the tef prolamin, being char-

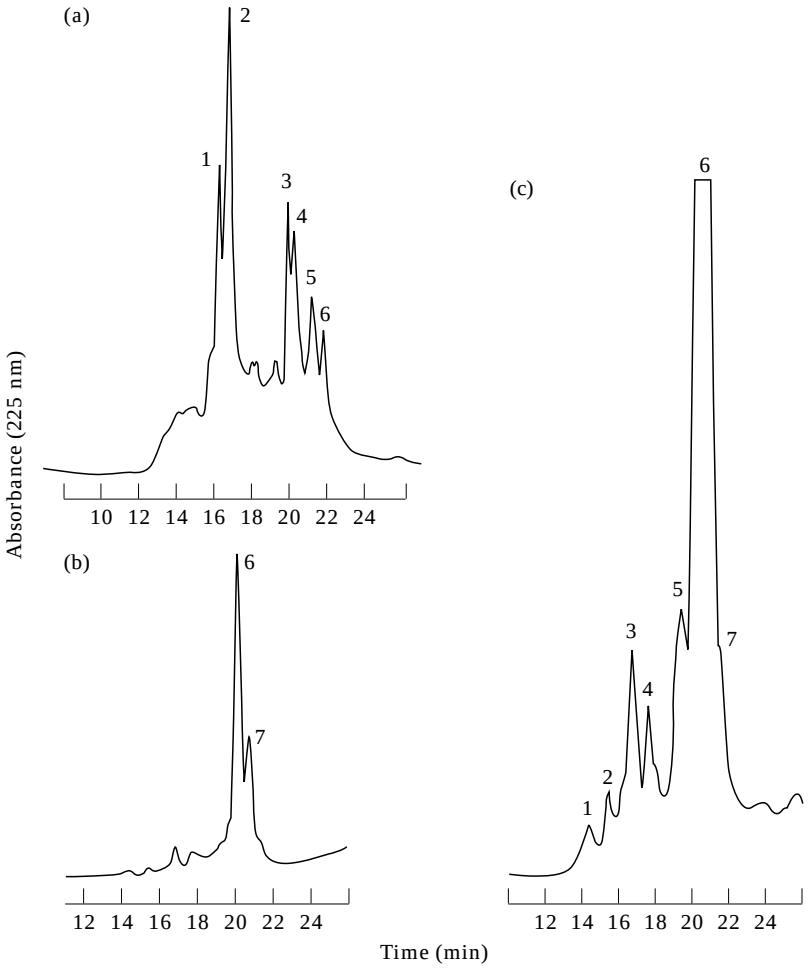

Figure 2 RP–HPLC profiles of tef and finger millet prolamins. (a) Tef prolamins. (b) finger millet prolamins and (c) as (b) but at higher loading. HPLC conditions described in text.

acterised by high contents of methionine and cysteine (20 mol% and 10 mol%, respectively). Tef peak 6 showed a weaker homology with the α-prolamins of the Andropogoneae and also had a deletion of 14 residues at the N-terminus compared with Z19 zein (Table II). Although peak 6 might, on the basis of its lower M_r, be expected to have shown greater homology with the Z19 prolamins of maize, this was not the case (Table II).

The HPLC-purified prolamins of finger millet were more heterogeneous than those of tef, and it was not possible to obtain long N-terminal sequences. However, the sequences obtained showed homology with those of tef, with finger millet peak 3 showing greater homology with tef peak 2, and finger millet peak 6 containing the characteristic PCV tripeptide sequence present in tef peak 6 (Table II). The finger millet prolamins also resembled the tef prolamins in having N-terminal deletions relative to the α-zeins and other prolamins of the Andropogoneae (data not shown).

Our studies indicate that the major prolamins of both tef and finger millet are similar to those of the α-prolamins of the Andropogoneae, notably maize, sorghum and *Coix*, as demonstrated by M_r, amino acid compositions and N-terminal amino acid sequences[15]. The α-prolamins consist of non-repetitive N- and C-terminal domains of about 36–37 and 10 residues, respectively, separated by a repetitive domain consisting of blocks of between about 14 and 25 residues (with an average length of 20 residues)[15], the size difference between the Z19 and Z22 groups of α-zeins resulting from the insertion of an additional repeat in the C-terminal domain of the Z22 protein. The N-terminal domain is clearly seen in the two tef sequences, with high

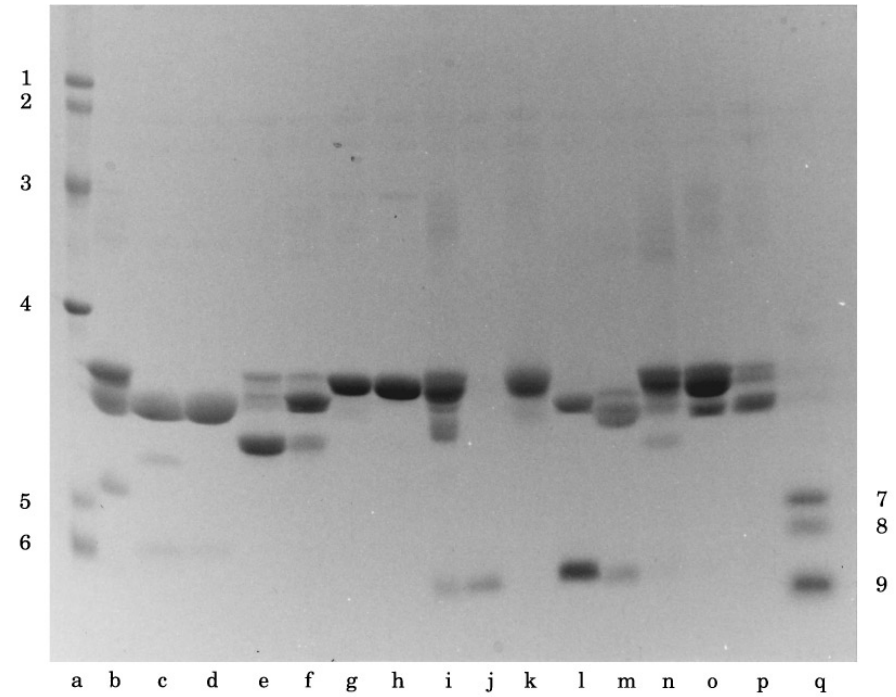

Figure 3 SDS–PAGE of RP–HPLC peaks of tef and finger millet. Track a = M_r markers (as Fig. 1 track a). Tef prolamins: track b = total; tracks c–h = peaks 1–6, as numbered in Fig. 2(a). Finger millet prolamins: track i = total finger millet; tracks j–p = peaks 1–7 as numbered in Fig. 2(b). Track q = M_r markers, as Fig. 1, track j.

Table I Amino acid compositions of selected tef and finger millet (FM) prolamins (mol%)

Amino acid	Tef 2	Tef 6	FM 3	FM 6	Total FM	α-zein
Asp + Asn	3·7	3·5	2·3	3·5	3·7	5·1
Glu + Gln	32·1	20·4	29·6	18·9	24·9	21·4
Ser	3·2	3·6	4·7	4·4	6·8	6·3
Gly	5·4	3·2	6·7	4·7	2·5	2·2
His	0·9	0·5	1·1	0·8	2·2	0·0
Arg	1·1	1·3	2·0	1·1	1·3	1·2
Thr	2·7	4·3	4·9	3·8	5·0	3·0
Ala	9·5	13·6	5·1	10·6	8·6	13·3
Pro	8·8	11·9	9·1	14·0	10·3	10·7
Tyr	3·7	5·8	4·0	3·8	2·9	3·5
Val	6·9	8·6	9·1	9·8	7·3	3·6
Met	2·8	2·8	5·0	2·0	2·2	0·9
Cys	1·4	1·1	1·1	1·4	1·2	1·0
Ile	4·3	4·1	3·7	4·5	4·4	3·8
Leu	7·9	6·7	7·3	7·5	10·6	18·7
Phe	5·0	8·2	4·0	8·2	5·2	5·2
Lys	0·3	0·3	0·4	0·6	0·5	0·1

Tryptophan was not determined. Total finger millet prolamins calculated from the data of Ramachandra *et al.*[19] and α-zeins from Tatham *et al.*[16].

homology around the junction region with the first repeat of the repetitive domain (indicated in Table II). The sequences determined for the finger millet prolamins did not extend to this junction region.

Hilu and Esen[17], in a general survey of prolamins in the Poaceae, found that the Panicoideae and Chloridoideae had prolamins of one major size group, between about M_r 20 000–26 000; later,

Table II N-terminal amino acid sequences of selected tef and finger milled prolamins. (a) N-terminal sequences, (b) alignment of tef sequences, (c) alignment of tef 2 with Z22/Z19 sorghum, *Coix* and rice sequences. The arrow marks the beginning of the host repeat. | = sequence homology; 3 = homology: single base change.

(a)

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Tef 2      VTFPQYFPSXTPLAXNSPYXQYY
           VTFPQYFPSCTPLAINNPYVQYYPLQQAFT

Tef 6      YHTAFHYPQFPLASVPCVQYATLQQVMAXGI

FM 3       VIFPQFIPSX
                Y

FM 6       VNYLQYPSPFLAFYVIIPCV
                     V  T ASN
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(b)

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Tef 2      VTFPQYFPSCTPLAINNPYVQYYPLQQAFT
Tef 6       YHTAFHYPQFPLA-SVPCVQYATLQQVMAXGI
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(c)

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Zein Z22   SIIPQCSLAPSAIIPQFLPPVTSMAFEHPAVQAYRLQQALA
Tef 2                VTFPQYFPSCTPLAINNPYVQYYPLQQAFT
Zein Z19   TIFPQCSQAPIASLLPPYLSPAVSSVCENPILQPYRIQQAIA
                                                 ↑

Sorghum    VIIPQCSLAPNAI-IPQFLPPLTPVGFEHPALQAYRGQQALA
Tef 2                VTFPQYFPSCTPLAINNPYVQYYPLQQAFT
Coix       VIIPQCSLAPTAAIIPRFLPHVSAIGFEHPALQAYRLQQALA
                                                 ↑

Tef 2      VTFPQYFPSCTPLAINNPYVQYYPLQQAFT
Rice 10kD  ITTMQYFPPTLAMGTMDPCRQYMMQTLGMG

Tef 6                    YHTAFHYPQFPLASVPCVQYATLQQVMAXG
Zein Z19   TIFPQCSQAPIASLLPPYLSPAVSSVCENPILQPYRIQQAIAAG
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more detailed studies of prolamin patterns in the Chloridoideae[13] and Panicoideae[18] confirmed these observations.

The results reported here demonstrate that the prolamins of tef (*Eragrostis tef*) and finger millet (*Eleusine coracana*) are closely related to the α-prolamins of the Andropogoneae (maize, sorghum and *Coix*), despite being classified in different sub-families of the Poaceae (the Chloridoideae).

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