Characterization of the Storage Protein in Seed of *Coix lacryma-jobi* var. Adlay

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The endosperm of seed of *Coix lacryma-jobi* var. Adlay contains ca. 20% protein distributed between fraction 1 (albumins and globulins), fraction 2 (prolamins), and fraction 3 (residual proteins) extracts. The major component is prolamin, known as coixin, the amount of which ranged from 8.4% to 78.7% of the total endosperm protein depending upon the concentration of 2-mercaptoethanol in the extraction solvent. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis separated coixin into five components with molecular weights of 27K (C1), 25K (C2), 22K (C3), 17.5K (C4), and 15K (C5). Isoelectric focusing (IEF) of coixin resolved seven major protein bands. Analysis of the individual IEF bands by SDS-PAGE and immunoblotting, using rabbit antibodies raised against C2 and C3, indicated an even greater complexity, with a total of 17 proteins being detected. Amino acid analysis revealed that protein fractions from *Coix* endosperm have the typical composition of Panicoideae endosperm. The predominant fraction, coixin, is rich in proline and leucine and poor in lysine.

Coix lacryma-jobi L. is the most widely distributed of four *Coix* species belonging to the family Coicineae of the grass tribe Andropogoneae (Clayton, 1973, 1983; Jain and Banerjee, 1974). *Coix* is native to India, Burma, China, and Malaysia and was grown extensively in South Asia before maize became popular as an agricultural crop (Arora, 1977). There are a number of varieties differing in plant size and seed structure. For instance, seed of wild forms have a hard shell while some cultivated varieties are characterized by a soft-shelled seed (Arora, 1977). Over the years *Coix* has been used as a food source for humans (Wester, 1920; Schaaffhausen, 1952) and livestock (Raimo and Leme, 1950; Torres and Bergamin, 1951). It has also been utilized in the production of alcoholic beverages and as a medicinal plant (Ridley, 1906; Arora, 1977).

C. lacryma-jobi var. Adlay was introduced into Brazil around 1939 and was subsequently subjected to breeding programs to improve agronomic characteristics such as yield and growth period (Schaaffhausen, 1952). The seed contains around 20% protein, the major constituent of which is a prolamin called coixin (Venkateswarlu and Chaganti, 1973). In this paper we report on an analysis of the storage proteins of *C. lacryma-jobi* var. Adlay in which special attention was centered on the electrophoretic and immunological properties of two of the predominant coixin proteins.

MATERIALS AND METHODS

Plant Material and Protein Extraction. *C. lacryma-jobi* var. Adlay seed was obtained from the collection of the Genetics Department, University of Campinas. Endosperms, obtained by hand dissection of 500 seeds, were ground to a flour and defatted with acetone. The endosperm proteins were extracted sequentially with use of the procedures outlined in Table I. Nitrogen determinations on the resultant F1, F2, and F3 protein fractions were carried out according to Nkonge and Ballance (1982).

Table I. Procedures for the Sequential Extraction of Proteins from the Endosperm of Seed *C. lacryma-jobi* var. Adlay

fraction	extraction conditions	proteins
F1	extract twice with 0.5 M NaCl (5:2, v/w) for 1 h at 4 °C	albumins, globulins, free amino acids
F2	after extracting F1 proteins, extract with 4:1 2-propanol (v/w) overnight and 3:1 2-propanol (v/w) for 2 h at 25 °C in the presence of varying amounts of 2-ME (0–20%)	prolamin (coixin)
F3		residual proteins

Amino Acid Analysis. Approximately 100-μg aliquots of F1, F2, and F3 proteins were hydrolyzed with 6 N HCl at 110 °C for 20 h. The hydrolyzed samples were neutralized with NaOH and lyophilized. The samples were dissolved in 200 μL of distilled water in a glass V-vial and mixed with 50 μL of 1 M borate buffer (pH 6.3) and 200 μL of 15 mM 9-fluorenylmethyl chloroformate (FMOC). After 5 min at room temperature excess reagent was removed by partitioning three times against equal volumes of pentane (Einarson et al., 1983). The fluorescent FMOC–amino acid derivatives were analyzed by high-performance liquid chromatography (HPLC) as described by Nasholm et al. (1987).

Electrophoretic Methods. All three protein fractions were analyzed by 5–20% SDS-PAGE gradients according to Laemmli (1970). Electrophoresis was carried out at 100 V at room temperature after which gels were stained for 2 h with 0.25% Coomassie Brilliant Blue R250 in methanol/water/acetic acid (5:5:1, v/v) and subsequently destained in methanol/water/acetic acid (3:6:1, v/v). Estimates of molecular weight were based on the procedures of Plikaytis et al. (1986).

IEF of the F2 proteins was carried out on agarose gels with pH 5–8 ampholines as described by Wilson (1984). The gels were 0.75 mm thick and contained 1% agarose, 0.4% pH 3.5–9.5 ampholine (LKB, Bromma, Sweden), and 1.6% pH 5–8 ampholine (LKB) in 5 M urea and 2 mM dithiothreitol. After

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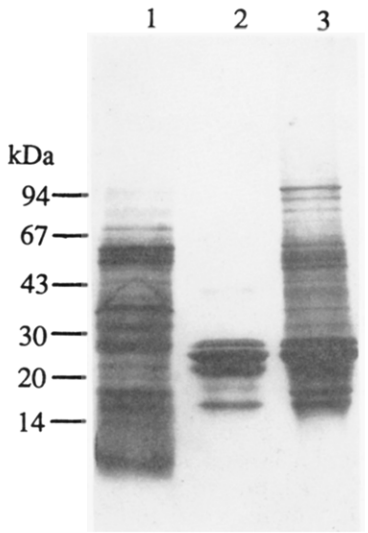

Figure 1. SDS-PAGE analysis of F1, F2, and F3 proteins from 500 mg of endosperm of *C. lacryma-jobi* var. Adlay: lane 1, F1; lane 2, F2; lane 3, F3. Proteins stained with 0.25% Coomassie Brilliant Blue R250 in methanol/water/acetic acid (5:5:1, v/v). Arrows indicate positions of protein standards of known molecular weights. The residual proteins (F3) were solubilized by adding (3:1, v/w) 100 mM Tris-HCl (pH 7.5), 5% SDS, and 7.5% 2-ME for 2 h followed by boiling for 5 min.

Table II. Distribution of Protein in F1, F2, and F3 Extracts from the Endosperm of Seeds of *C. lacryma-jobi* var. Adlay

2-ME in F2 solvent, %	protein fraction	protein as % of endosperm	% of total endosperm protein
0	F1	0.5	2.7
	F2	2.0	10.8
	F3[a]	16.0	86.5
0.7	F1	0.5	2.4
	F2	8.8	41.9
	F3	11.7	55.7
5.0	F1	0.5	2.8
	F2	12.3	68.3
	F3	5.2	28.9
10.0	F1	0.4	1.9
	F2	15.2	71.7
	F3	5.6	26.4
20.0	F1	0.4	1.8
	F2	16.8	77.8
	F3	4.4	20.4

[a] The residual proteins (F3) were stimated by digesting the endosperm residue after F1 and F2 extraction.

focusing, gels were fixed in acetic acid/trichloroacetic acid/water (5:3:92, v/v) and stained with 0.5% Coomassie Brilliant Blue R250 in methanol/water/acetic acid (2:7:1, v/v). Focusing destained gels were dried at room temperature. The isoelectric point (p*I*) of each IEF band was determined by reference to protein standards (Kit No. IEF-MI; Sigma, St. Louis, MO). For sequential analyses, the IEF gels were treated with trichloroacetic acid/acetic acid/water (3:5:92, v/v) for 3–4 h to precipitate proteins. The gels were then washed with water and dried with a hair drier and individual protein bands cut out and analyzed by SDS-PAGE to provide two-dimensional-equivalent resolution (Wilson, 1986) of the major coixin polypeptides.

Purification of Coixins. The coixin fraction was subjected to preparative 12% SDS-PAGE according to the procedures of Laemmli (1970). Gels were 5 mm thick, and samples consisted of 4 mg of protein dissolved in 2 mL of loading buffer. Electrophoresis was carried out at room temperature at 100 V until the tracking dye reached the end of the gel. The gels were rinsed in cold water and then treated with 0.25 M KCl for 5 min to visualize protein bands (Hager and Burgess, 1980). Two of the major proteins, C2 and C3, were cut out and recovered from gel slices by electroelution, after which they were lyophilized, resuspended in 55% aqueous isopropyl alcohol containing 2% 2-ME, and stored at −20 °C. Analyses of the C2 and C3 samples by SDS-PAGE revealed the presence of single bands in both instances.

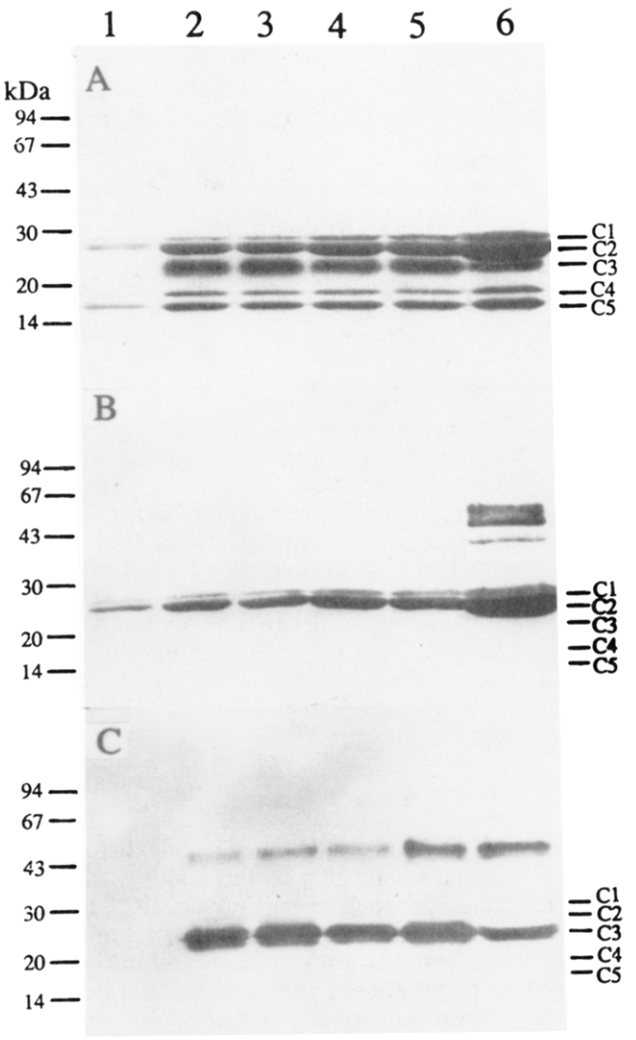

Figure 2. SDS-PAGE analysis of F2 coixins from 500 mg of endosperm of *C. lacryma-jobi* var. Adlay extracted with 55% aqueous isopropyl alcohol containing 0% (lane 1), 0.7% (lane 2), 2% (lane 3), 5% (lane 4), 10% (lane 5), and 20% 2-ME (lane 6): A, stained with 0.25% Coomassie Brilliant Blue R250 in methanol/water/acetic acid (5:5:1, v/v); B, immunoblotting analysis of replicate gel (proteins transferred to nitrocellulose membrane, incubated with C2 protein antiserum for 1 h, washed and then treated with horseradish peroxidase linked protein A for 1 h and stained by immersion in color indicator solution); C, as (B) but with C3 protein antiserum. Arrows indicate positions of protein standards of known molecular weights (left) and C1–C5 protein bands (right).

Preparation of Antibodies. Antibodies were raised against the purified coixin proteins C2 and C3 in New Zealand white rabbits. The animals were injected subcutaneously with 1 mg of protein in 1 mL of 55% aqueous isopropyl alcohol emulsified with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI). Fifteen days later, the rabbits received a booster injection in which the protein solution was emulsified with Freund's incomplete adjuvant (Difco). After a further 15 days, blood was collected and the antisera were decanted and stored at −20 °C. The titre of the antisera was determined by an ELISA procedure using an N812 kit (Amersham International, Amersham, Bucks, U.K.).

Western Blot Analyses. Following SDS-PAGE, coixin proteins were transferred to BA-85 nitrocellulose sheets (Schleicher and Schuell, Bassel, German Federal Republic). The nitrocellulose membranes were then incubated on a rotary shaker for 1 h in Tris-buffered saline (pH 7.6) (20 mM Tris-HCl, 137

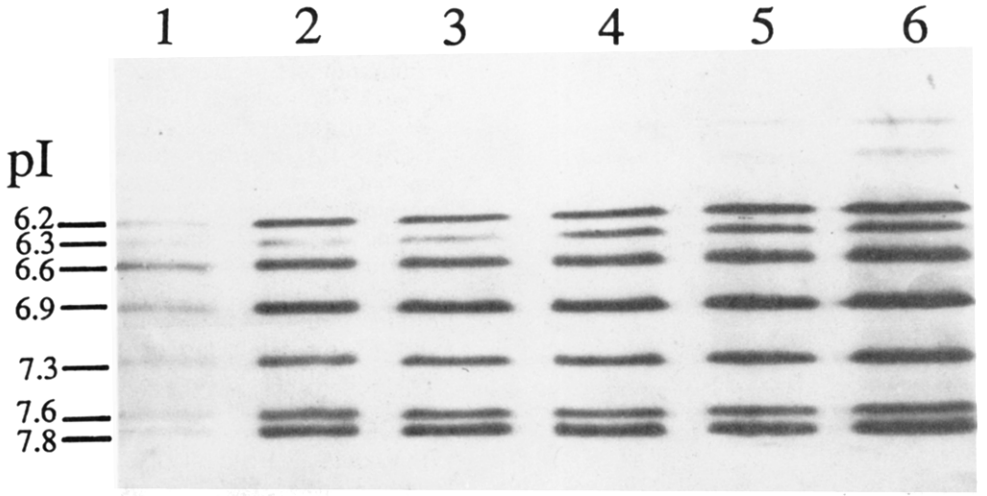

Figure 3. IEF of F2 coixins from 500 mg of endosperm of *C. lacryma-jobi* var. Adlay extracted with 55% aqueous isopropyl alcohol containing 0% (lane 1), 0.7% (lane 2), 2% (lane 3), 5% (lane 4), 10% (lane 5), and 20% 2-ME (lane 6). Proteins stained with 0.5% Coomassie Brilliant Blue R250 in methanol/water/acetic acid (2:7:1, v/v). Arrows indicate p*I* values of protein bands.

Table III. Amino Acid Composition of F1, F2, and F3 Proteins Extracted from the Endosperm of Seed of *C. lacryma-jobi* var. Adlay As Determined by Ion-Pair Reversed-Phase HPLC of FMOC–Amino Acid Derivatives (Individual Amino Acid Levels Expressed as Mole Percent of Total Amino Acid Content; F2 Proteins Extracted with Solvent Containing 2% 2-ME)

	protein fraction		
amino acid	F1	F2	F3
aspartic acid	14.3	6.2	6.3
serine	6.9	5.4	6.8
glutamic acid	16.1	23.0	16.8
arginine	7.2	1.7	1.0
glycine	14.2	3.3	8.5
threonine	5.1	2.7	4.6
alanine	11.1	18.0	15.8
proline	5.1	9.9	9.5
methionine		0.2	1.8
valine	6.2	5.9	6.8
phenylalanine	2.7	4.1	3.5
isoleucine	3.0	3.4	3.6
leucine	4.2	15.8	12.4
lysine	2.8		0.8

mM NaCl) (TBS), 0.1% Tween-20, and 5% nonfat dried milk. Membranes were then transferred to 5% nonfat dried milk in TBS containing antiserum at a 1:1000 dilution and incubated overnight at 30 °C on a rotary shaker. Membranes were rinsed twice in 0.1% Tween-20 in TBS (TBS-T) and then washed three times for 10-min periods in TBS-T. The washed membranes were subsequently incubated for 1 h in a TBS solution containing a 1:5000 dilution of horseradish peroxidase linked protein A (Amersham International) after which they were immersed for 10 min in a color indicator solution (100 mL of 50 mM acetate buffer (pH 4.5), 50 mg of 3-amino-9-ethylcarbazole in 3 mL of dimethylformamide, 100 μL of H_2O_2). When the desired degree of staining was achieved, the reaction was stopped by washing membranes in distilled water.

RESULTS AND DISCUSSION

The F1, F2, and F3 storage proteins extracted from *C. lacryma-jobi* var. Adlay composed ca. 20% of the dry weight of the endosperm (Table II), which is about double that of other cereals (Payne, 1983). As endosperm was extracted with increasing amounts of 2-ME in the F2 extraction medium, there was a marked rise in the F2 coixins and a concomitant decrease in the F3 residual proteins. When the concentration of 2-ME was ≥5%, coixins represented the major protein fraction.

The F1, F2, and F3 proteins were analyzed by SDS-PAGE. The F1 extract was complex, containing protein bands with apparent molecular weights ranging from 9 to 110K (Figure 1, lane 1). The F2 coixins consisted of five major bands, namely C1 (27K), C2 (25K), C3 (22K), C4 (17.5K), and C5 (15K) (Figure 1, lane 2). The F3 sample was very heterogeneous and contained significant amounts of coixins. The molecular weight range of the F3 proteins was 15–110K (Figure 1, lane 3). These observations and the data in Table II imply that the coixins are only extracted efficiently from *Coix* endosperm when the level of 2-ME in the F2 solvent is raised to 20%. An analogous situation has been reported in maize with respect to the extraction of the prolamin, zein, and it has been suggested that zein components may be trapped in the residual protein matrix or possibly aggregated with residual protein molecules by disulfide bonds (Lee et al., 1976).

The amino acid composition of all three *Coix* protein fractions, as determined by ion-pair reversed-phase HPLC of the FMOC derivatives, is shown in Table III. The F1 protein amino acid content is similar to that of the soluble protein and free amino acid fraction commonly associated with cereals (Sodek and Wilson, 1971; Bayers et al., 1983; Miflin et al., 1983), with glutamic acid, glycine, and alanine as the main constituents. The amino acid profile of the coixins (F2) appears to be that of the zein family (Wilson, 1983) characterized by an absence of lysine and the presence of relatively large amounts of glutamic acid, alanine, proline, and leucine and found in all members of the Panicoideae investigated to date. The F2 protein was extracted in the presence of 2% 2-ME, and as a consequence the F3 sample would contain significant amounts of residual coixins (see Figure 1). This is reflected in the fact the amino acid compositions of the F2 and F3 extracts are very similar (Table III).

SDS-PAGE analysis of the F2 extract demonstrated that increasing the amount of 2-ME in the extraction solvent enhanced the yields of the C1, C2, C4, and C5 proteins (Figure 2A). The use of higher concentrations of 2-ME resulted in the appearance of high molecular weight protein bands. Western blot analyses showed that C2 antiserum recognized both C1 and C2 proteins and cross-reacted with high molecular weight components (39–52K) extracted only in the presence of 20% 2-ME (Figure 2B). C3 antibodies reacted with the C3 band and cross-reacted with a 40.5K protein, the levels of which,

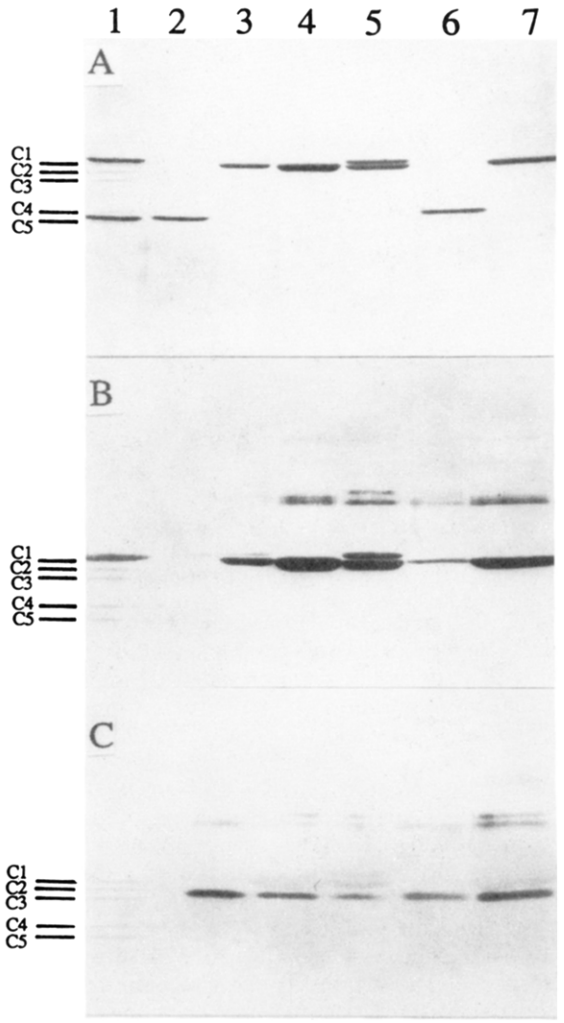

Figure 4. SDS-PAGE analysis of individual coixin IEF proteins: lane 1, 7.8 p*I* band; lane 2, 7.6 p*I* band; lane 3, 7.3 p*I* band; lane 4, 6.9 p*I* band; lane 5, 6.6 p*I* band; lane 6, 6.3 p*I* band; lane 7, 6.2 p*I* band. Key: A, gel stained with 0.25% Coomassie Brilliant Blue R250 in methanol/water/acetic acid (5:5:1, v/v); B, immunoblotting analysis of replicate gel (proteins transferred to nitrocellulose membrane, incubated with C2 protein antiserum for 1 h, washed, treated with horseradish peroxidase linked protein A, and stained by immersion in color indicator solution); C, as (B) but with C3 protein antiserum. Arrows indicated positions of C1–C5 coixins.

in contrast to C3, increased with increasing concentrations of 2-ME (Figure 2C). The C3 protein was not detected in the absence of 2-ME and increased in size only marginally as the level of reducing agent increased (Figure 2A,C). In this respect, the behavior of the C3 coixin is similar to that of the maize prolamin γ-zein (Esen, 1986), which is classified as a reduced soluble protein (Vitale et al., 1982; Larkins et al., 1984; Ludevid et al., 1985), although the former has a molecular weight of 22K and the latter 27K.

The response of the C1 band to C2 antiserum (Figure 2B) could be a consequence of the two proteins having common epitopes. In view of the close proximity of the C1 and C2 bands on the SDS-PAGE gel used for purification prior to immunization (see Figure 1, lane 2), it is also possible that the C2 antigen was contaminated with C1 protein. However, cross-reactivity between the coixin polypeptides is so extensive that this is unlikely.

IEF of F2 from *Coix* endosperm revealed the presence of seven major bands with p*I* values ranging from 6.2 to 7.8. The intensities of the seven bands increased as the amount of 2-ME in the extraction medium was increased (Figure 3). All seven IEF bands were analyzed individually by SDS-PAGE, with replicate gels being subjected to immunoblotting, using C2 and C3 antisera, and staining with Coomassie Brilliant Blue. The data obtained are illustrated in Figure 4, examination of which reveals that SDS-PAGE subdivided the IEF bands into several components, demonstrating that the IEF bands were not single polypeptides. Consideration of the SDS-PAGE staining (Figure 4A) and immunoblotting data (Figure 4B,C) indicate that the C1–C5 SDS-PAGE coixins are composed of at least 17 polypeptides. There are four C1 proteins, both C2 and C3 consist of five individual bands, and C4 is a single component, while C5 is composed of two polypeptides.

C3 is not detected as a major component in any of the seven IEF coixin bands when the SDS-PAGE gel is stained with Coomassie Brilliant Blue (Figure 4A). However, C3-type proteins are clearly evident in five of the IEF bands when the gel is subjected to Western blot analysis using C3 antiserum (Figure 4C). Similarly, several of the IEF bands contain high molecular weight proteins that were not evident when the SDS-PAGE gel was stained but were detected by immunoblotting with C2 and C3 antisera.

ABBREVIATIONS

FMOC, 9-fluorenylmethyl chloroformate; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; 2-ME, 2-mercaptoethanol; p*I*, isoelectric point; TBS, Tris-buffered saline; TBS-T, 0.1% Tween-20 in Tris-buffered saline.

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