One- and Two-Dimensional Gel Electrophoresic Identification of African Yam Bean Seed Proteins

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Seed proteins were extracted from the African yam bean (AYB; *Sphenostylis stenocarpa*), an underutilized West African food legume. One- and two-dimensional polyacrylamide gel electrophoresis was then used to analyze the albumin fraction, galactose-specific lectins purified on immobilized galactose−Sepharose 4B, and abundant non-lectin seed proteins left over following affinity chromatography. N-terminal sequencing of prominently resolved polypetide bands led to identification of proteins having sequence homology with characterized legume seed proteins, namely, mung bean seed albumin, pea α-fucosidase, soybean Kunitz-type trypsin inhibitor, an endochitinase, pea pathogenesis-related protein, and/or cowpea seed storage proteins. Minor lectin-like proteins lacking hemagglutinating activity against rabbit and human erythrocytes were also identified. Because proteins such as protease inhibitors, chitinases, pathogenesis-related proteins, and lectins are known to have antimetabolic effects, the findings from this study may have relevance in the acceptability, adoption, and utilization of AYB as human food.

Keywords: *albumins; electrophoresis; lectins; N-terminal protein sequencing; seed proteins; Sphenostylis stenocarpa*

INTRODUCTION

Despite the high yield and nutritional value of *Sphenostylis stenocarpa* [(Hoechst ex A. Rich) Harms], commonly known as African yam bean (AYB), this underutilized crop has failed to gain popularity comparable to that of other edible African legumes such as cowpea and pigeon pea (Okigbo, 1973; Potter, 1992). The lack of acceptability and adoption over the years may be partly attributed to the presence of toxic antinutritional secondary metabolites such as saponins, flavonoids, and alkaloids, which are known to be present in seeds and vegetative parts of this plant (Asuzu and Undie, 1986). Research at the International Institute of Tropical Agriculture (IITA) based at Ibadan, Nigeria, has shown that AYB is more resistant to cowpea pests, particularly the legume pod borer, *Maruca vitrata*, than other edible legumes such as cowpea (*Vigna unguiculata*). In seeds, primary metabolites, particularly lectins and protease inhibitors, are likely to contribute more to their antinutritive properties than secondary metabolites. In particular, dietary lectins are highly resistant to gut proteases and have the ability to bind to glycan receptors on the surface of the epithelium along the gastrointestinal tract of animals and humans, thus causing a noxious effect (Pusztai and Bardocz, 1996).

Although the nutritional value of AYB has been well studied (Evans and Boulter, 1974; Nwokolo, 1987; Oshodi et al., 1995), very little is known about the existing levels of primary metabolites in AYB. For this reason, we have recently identified and purified galactose-specific AYB seed lectins using affinity chromatography (Machuka et al., 1999). The objective of the present study was to continue the characterization of AYB seed lectins and to identify specific proteins that may have implications in the utilization and processing of AYB for human consumption, as well as in crop protection strategies utilizing plant defense proteins.

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EXPERIMENTAL PROCEDURES

Plant Material and Reagents. On the basis of differences in seed color, AYB seeds were collected from local markets in Umuahia and Enugu in eastern Nigeria and successfully multiplied at the IITA, Ibadan, Nigeria, between October 1996 and March 1999. Seeds were harvested and stored at 4 °C until needed for protein extraction. All reagents were of analytical grade. Phenyl-Sepharose, Sepharose-4B, SP Sepharose Fast Flow, Sephadex G25, and Sephacryl S300 were purchased from Pharmacia (Uppsala, Sweden). 1,3-Diaminopropane (DAP) and polyvinyl sulfone were from Sigma (Germany).

Lectin Affinity Chromatography. Seed meal from four AYB accessions (EN953, EN971, EN982, and UM971) was extracted and used for lectin affinity chromatography as previously described (Machuka et al., 1999). Galactose-derivatized Sepharose-4B affinity matrix was prepared according to a divinyl sulfone coupling method (Peumans et al., 1997). An ammonium sulfate precipitate was loaded onto a column of galactose−Sepharose-4B equilibrated with 2 M ammonium sulfate containing 0.2 M galactose. Lectin eluted from this column was concentrated with phenyl-Sepharose, desalted with Sephadex G25, dialyzed, and lyophilized.

Fractionation of Seed Albimins. Seed albumins were fractionated using a modification of the procedure described by Rüdiger (1993). Seed meal was extracted in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ for 2 h at 4 °C. The extract was filtered through Miracloth and centrifuged at 23500*g* for 20 min at 4 °C. Seed storage proteins were precipitated by slowly adding 1 M acetic acid until pH 4.5 was reached and stirring the solution for at least 1 h at 4 °C. The suspension was centrifuged again at 23500*g* for 20 min at 4 °C. The albumin-enriched supernatant solution was adjusted to pH 8.0 with 1 M NaOH, dialyzed

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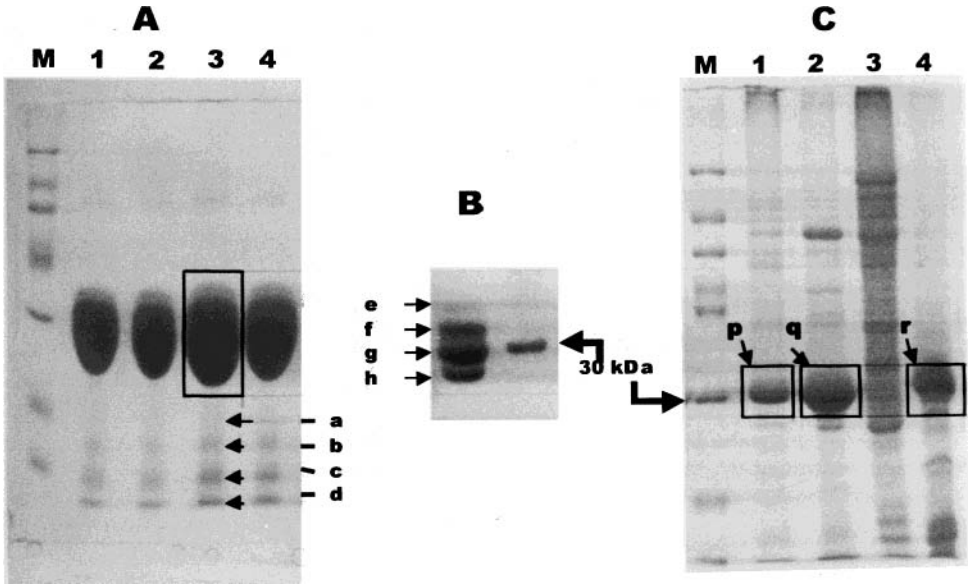

Figure 1. SDS−PAGE (12%) of *S. stenocarpa* lectin (A, B) and non-lectin (C) fractions. Wells contain 100 μg (A) and 40 μg (B) each of purified lectin and 100 μg (C) of unbound protein following affinity chromatography. Low molecular weight non-lectin bands eluted from galactose column (bands a−d in A) and the most prominent unbound polypeptide bands (bands p−r in C) are indicated. Purified lectin in (B) is from EN982 (track 3, boxed). Molecular mass reference proteins (M), in order of increasing molecular mass, are lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5/19.7 kDa doublet), carbonic anhydrase (31 kDa), aldolase (40 kDa), ovalbumin (42.7 kDa), glutamate dehydrogenase (55 kDa), bovine serum albumin (66 kDa), and phosphorylase B (97.4 kDa). Lane 1, EN953; lane 2, EN971; lane 3, EN982; lane 4, UM971.

against water, and lyophilized. The procedures for total protein extraction and quantitation and hemagglutination assays using rabbit and human erythrocytes were as described (Machuka et al., 1999).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was carried out using 12% acrylamide gels (Laemmli, 1970). Gels were stained either with Coomassie Brilliant Blue R-250 or with Bio-Rad's copper stain and destain reagent solution and calibrated using the midrange protein molecular weight standards (Promega, Madison, WI; and Sigma, St. Louis, MO).

Two-Dimensional (2D) Electrophoresis. Two-dimensional electrophoresis was carried out in the Mini-Protean II 2D cell (Bio-Rad, Hercules, CA). Isoelectric focusing was done in 7.5 cm long capillary tubes that were 1.0 mm in diameter. The gel composition was 4% acrylamide, 9.2 M urea, 20% Triton X-100, 1.6% Bio-Lyte 6.4/7.1 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonium persulfate, and 0.1% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). The anolyte was 100 mM sodium hydroxide, and the catholyte was 10 mM phosphoric acid. Prior to loading, 100 μg of protein (in 10 μL volume) was incubated for 30 min with first-dimension sample buffer [9.5 M urea, 2.0% Triton X-100, 5% 2-mercaptoethanol (2-ME), 1.6% Bio-Lyte 6.4/7.1 ampholyte, and 0.4% Bio-Lyte 3/10 ampholyte]. Focusing was carried out for 4 h at 750 V. The gels (7.0 mm × 10.0 mm long and 0.1 mm thick) were equilibrated for 30−35 min in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 5.0% (v/v) 2-ME, 10% glycerol (w/v), and 0.0012% bromophenol blue (w/v). The second dimension utilized Bio-Rad's vertical slab gel apparatus, model 220, with 12% acrylamide gels containing 10% (w/v) SDS and 1.5 M Tris-HCl, pH 8.8. After focusing, gels were washed overnight in 15% (w/v) trichloroacetic acid. They were then stained with Coomassie Brilliant Blue R-250.

Determination of pH Gradients. The pH gradient across one-dimensional (1D) gels was measured according to the method of Gatehouse et al. (1980). Slices were removed from the focused gels and sectioned, and the gel pieces (1 mm × 10 mm) were soaked overnight in 400 μL of distilled water. The pH values of the solutions were then measured.

N-Terminal Protein Sequencing. For N-terminal protein sequencing, polypeptide bands were separated by SDS−PAGE and electroblotted onto poly(vinylidene difluoride) (PVDF) membrane. The desired bands were then excised and sequenced on an Applied Biosystems model 477A ABI protein sequencer with a 120A analyzer.

RESULTS AND DISCUSSION

Affinity-purified lectins were visualized on SDS−PAGE gels as shown in Figure 1A,B. The eluted fraction always contained low molecular weight (LMW) bands that migrated at approximately 6.5, 11, 18, and 21 kDa, respectively. These bands were visualized clearly on SDS−PAGE gels only when >100 μg of purified lectin was loaded (bands a−d in Figure 1A). To achieve a pure lectin preparation, a size exclusion chromatography step using Sephacryl S300 (Pharmacia, Uppsala, Sweden) was necessary to remove trace amounts of the LMW polypeptides (Machuka et al., 1999). SDS−PAGE analysis of unbound protein following affinity chromatography showed that the predominant polypeptides in the seed protein fraction were in the size range of 26−34 kDa (Figure 1C). Preliminary quantitative analysis indicated that lectins alone account for 25−31% of total protein per mature, dry seed. This estimate is not surprising, considering that some lectins, such as phytohemagglutinin from *Phaseolus vulgaris*, account for up 50% of the total seed protein (Sharon and Liss, 1990).

The predominant band in the albumin fraction observed on 1D gels was resolved into more than eight bands on 2D gels (Figure 2A). The four lectin subunits (M_r = 34, 32, 29, and 27 kDa for bands e, f, g, and h in Figure 1B, respectively) had identical isoelectric points (pI = 5.1−5.4), unlike the other proteins which had different pI values, mostly in the acidic range (Figure 2A,C). N-terminal sequencing of the LMW polypeptides (bands a−d in Figure 1A) revealed similarities to the previously characterized lectins for at least five amino acid residues, namely, DNFFS. Comparison of the sequence of one LMW polypeptide (band b in Figure 1A) and sequences of lectin subunits (bands e and f in Figure

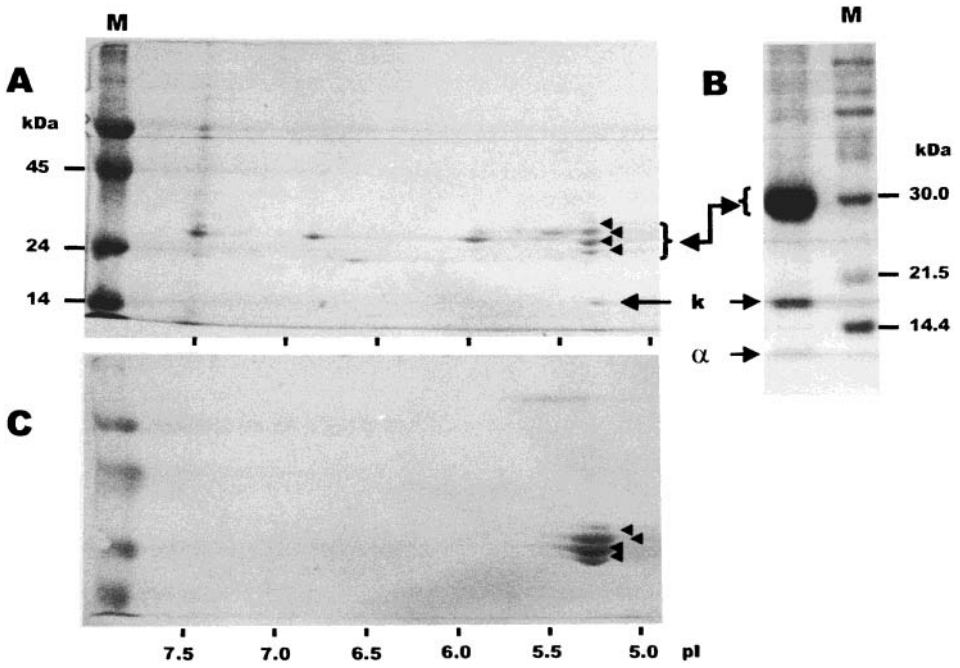

Figure 2. 2D (A, C) and 1D (B) PAGE of *S. stenocarpa* (EN953) albumins (A, B) and affinity-purified lectins (C). Lectin subunits are indicated with arrowheads. Corresponding positions of prominent polypeptides (26−34 kDa) in the albumin fraction on 1D and 2D gels are shown by an elbow double-arrow connector. Approximate isoelectric points (p*I*) for the various polypetides are also shown. M, molecular weight reference marker.

Table 1. N-Terminal Amino Acid Sequences of African Yam Bean Proteins and Comparisons to Other Legume Protein Sequences

Species/ Accession	Fig. No.-Band or Acc. No.*	Amino acid Sequence†	Identity/homology§
EN982	1A-a	DNFFF??????DSP	LLP
	1A-b	DNFFSFGIKNFDSPKRDLDF	LLP
	1A-c	DNFFS	LLP
	1A-d	DNFF	LLP
	1B-e	DNFFSFGIKNFSSDDLILQS	LS
	1B-f	DNFFSFGIKNFSSDDLILQS	LS‡
	1B-g	DNFFSF	LS
	1B-h	DNFFSF	LS
Pea	J01254	DIYFNFQRFNETSDNLILQR	seed lectin
UM971	1C-r	AYIDYAPGTTNDKILSGPAT	seed albumin
Mung bean	S58127	AYIDYAPGTTNDKILAGPTT	seed albumin
Pea	P08688	--LDYAPGTSNDKVLYGP--	major seed albumin
UM971	1C-r	KNCENLADDYYGGPCFTN	SP or PR-p
	1C-r	KNCENLADTYRGPCFTT	SP or PR-p
EN971	1C-q	KNCENL	SP or PR-p
Cowpea	P18646	KTCENLVDTYRGPCFTTG	SP
Pea	L01579	NTCEHLADTYRGVCFTNA	PR-p
EN971	1C-q	EKEKPGIAVYWGENPGDGTL	endochitinase
	1C-q	--DDASIAVYW???KYELDLR	endochitinase?
EN953	AF137070	FALEFALAVYWGENGGEGTL	endochitinase
EN9612	2B-k	KDDAEPVFDVGGNPLQLGGKYYI?M	SP/SKTI?
Winged bean	A61491	-DVAEDVLDIGGNPLLLGGEYY1	SP
Soybean	X80039	---ADIVFDTEGNPIRNGGTYYV	SKTI
EN9612	2B-α	PGVIFTGVTPLDIEFTKKPNNAENR	α-fucosidase
Pea	X82595	PGIIFTG-TPIEIEYTKKPNCAKS	α-fucosidase

*Genebank, EMBL and SwissProt database accession numbers
‡N-terminal sequence of this subunit was reported in Machuka *et al.* (1999).
†?, information uncertain; -, gaps introduced to maximize alignment.
§LLP, lectin-like protein; LS, lectin subunit; PR-p, pathogenesis-related protein; SP, seed protein; SKTI, soybean Kunitz trypsin inhibitor.

1B) shows clear differences between the two (Table 1). Previously, we have shown through hemagglutination assays that only the tetrameric hololectin agglutinates both rabbit and human erythrocytes (Machuka et al., 1999). In the present study, all other protein fractions, including the fraction containing only the LMW, lectin-like polypeptides (LLPs) described above, did not agglutinate trypsinized and untrypsinized rabbit and human erythrocytes. One possibility is that, like lectins, LLPs possess carbohydrate binding abilities. However, unlike lectins, they may be lacking agglutinating activities. An analogous situation exists in the genus *Phaseolus* for three related proteins, namely, phytohemagglutinin, arcelin, and α-amylase inhibitor (Mirkov et al., 1994).

The results of N-terminal sequencing of other bands that were resolved on SDS−PAGE gels are summarized in Table 1. Besides LLPs, at least eight other proteins were identified. Not surprisingly, most of these have homologies to classical seed storage proteins. For example, there are only two differences between the AYB protein and a mung bean albumin, that is, an alanine for a serine and a threonine for an alanine residue. As expected, the broad bands (p−r in Figure 1C) electroblotted for sequencing contained mixtures of several proteins. When the first few residues coincided with a sequence already identified, sequencing was terminated after the first five to seven residues to save costs. This is why only a few amino acid residues are shown in Table 1 for some bands. Another problem caused by mixtures of bands was poor sequencing runs that resulted in premature termination of the runs or sequences that posed difficulties in interpretation. Due to these reasons, it was impossible to obtain a meaningful sequence from band p.

Apart from classical seed storage proteins, polypeptide bands with homologies to several plant chitinases and α-fucosidases, soybean Kunitz-type trypsin inhibitor, and a pea pathogenesis-related (PR) protein were identified. Band r alone consisted of proteins that have close N-terminal sequence homology to one another, a cowpea seed protein and a pea PR protein. Band q (EN971) also consisted of a sequence for which the first few N-terminal amino acid residues (KNCENL) are identical to those obtained from sequencing of the albumin fraction from UM971. In addition, two other chitinase-like sequences were also revealed. The N-terminal endochitinase sequence (EKEKPGIAVYW-GENPGDGTL from accession EN971) has been used to design degenerate PCR primers for isolation and cloning of a full-length cDNA encoding an acidic endochitinase

from AYB (Colocci et al., 1999). Interestingly, the amino acid sequence predicted from the cDNA differs slightly from the two sequences generated by protein sequencing (Table 1). This is likely to be due to the existence of several related PR proteins (including chitinases) encoded by a multigene family (Levorsen and Chlan, 1999) rather than to sequencing errors. The existence of defense proteins such as protease inhibitors, lectins, LLPs, and a family of PR proteins is likely to explain the biochemical basis of resistance of AYB seeds to insect pests such as the cowpea weevil (*Callosobruchus maculatus*), pod-sucking bugs (*Clavigralla tomentosicollis*), and the legume pod-borer (*Maruca vitrata*) (Omitogun et al., 1999). As with lectins, it is essential to purify the proteins identified by N-terminal sequencing to homogeneity to study their effects, either singly or in combination, for example, on insect development. As a case in point, we have now shown that affinity-purified AYB galactose-specific lectins have antiinsect activities against larvae of the cowpea weevil but not against larvae of the pod borer (Machuka et al., 2000).

CONCLUSIONS

Using a combination of gel electrophoresis and N-terminal sequencing, we have identified several potentially antinutritional proteins from the seeds of AYB. These proteins may have significant implications in the processing and utilization of AYB seed for human consumption. For example, extensive soaking and boiling requirements normally applied to seed prior to consumption may be necessited by the presence of toxic proteins such as lectins, protease inhibitors, and chitinases. Current research efforts aim to utilize the amino acid sequences reported here to isolate genes encoding these proteins and to purify and study the biological properties of these proteins and their effects on the development of key cowpea pests and fungal pathogens.

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