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Characterization of the seed proteins of velvet bean (*Mucuna pruriens*) from Nigeria

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Abstract

This paper reports the preliminary characterization of seed protein fractions from seven varieties of velvet beans (*Mucuna pruriens*) grown in Nigeria, using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Three of the most abundant polypeptides, with approximate Mr of 23, 26 and 30 kDa, respectively, were further separated by preparative native-PAGE. N-terminal sequencing revealed the presence of the consensus sequence DDREPV-DT–PL that is also present in the soybean Kunitz-type trypsin inhibitor. The albumin fraction was also shown to contain both trypsin and chymotrypsin inhibitors through enzyme inhibitor assays. Western analysis using antibodies, raised against a representative, 23 kDa polypeptide, indicated that this protein species accumulates exclusively during seed development, suggesting a role in seed storage. Haemagglutination assays using rabbit erythrocytes failed to detect the presence of lectins. The results are discussed within the context of the role of lectins and protease inhibitors in storage and plant defence. The findings are also relevant in view of the toxic and antimetabolic effects of these proteins, which determine the acceptability and adoption of velvet beans as animal and human feed. © 2000 Elsevier Science Ltd. All rights reserved.

1. Introduction

Velvet beans (*Mucuna* spp.) are herbaceous forage and food legumes that have for a long time found widespread usage as rotation crops for management of various pests and pathogens, as well as in soil improvement and weed control (Buckles, 1995; Duke, 1981). Seeds of velvet beans are known to produce the unusual non-protein amino acid 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA), a potent neurotransmitter precursor that is, at least in part, believed to be responsible for the toxicity of *Mucuna* seed (Lorenzetti, MacIsac, Arnason, Awang & Buckles, 1998). In West Africa, seeds require extensive boiling and soaking treatments to remove toxicity prior to cooking and consumption (Carsky, Tarawali, Becker, Chikoye, Tian & Sanginga, 1998; Kay, 1979). The presence and/or levels of toxic or inhibitory proteins may also underlie the biochemical basis of resistance of *Mucuna* to pests and pathogens that cause serious crop failure in other important legume crop species such as cowpeas. Furthermore, the presence of such proteins would limit the utilization of *Mucuna* in human and animal nutrition.

In legume seeds, globulins (7S vicilins and 11S legumins) account for 30–80% of the total seed protein, with albumins constituting the remainder (Derbyshire, Wright & Boulter, 1976). Typically, seed storage proteins occur as oligomers composed of two or more subunits that are in turn made up of a number of polypeptide chains. The 7S vicilins and 11S legumins found in legumes consist largely of multi-subunit complexes with molecular masses of 145–190 kDa and 320–400 kDa, respectively (Bewley & Black, 1994). The legumins consist of six non-identical subunits (52–65 kDa), with each subunit containing an acidic (33–42 kDa) and a basic (19–23 kDa) polypeptide. The seed storage proteins are laid down at a specific stage during seed development, principally to act as a store of nitrogen when the seedling germinates (Bewley & Black, 1994). Proteins within the albumin class are more diverse, both structurally and functionally. Within this class, lectins and protease inhibitors have attracted a lot of attention in recent years due to their defence role that renders them potentially useful in current crop protection strategies utilizing transgenic approaches (Reek, Kramer, Baker, Kanost, Fabrick & Behnke, 1997; Ward, Uknes & Ryals, 1994). *M. pruriens*

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from Brazil and India have very high levels of trypsin inhibitors in their seeds (Siddhuraju, Vijayakumari & Jarnardhanan, 1996; Udeliba & Carlini, 1998). Although lectins were detected in the Indian species, Udeliba and Carlini reported that these proteins were undetectable in the seeds of the same species grown in Brazil. This observation indicates that ecological and climatic factors may influence the levels and concentration of toxic and antinutritional factors in plants.

The objective of this study was to carry out a preliminary characterization of seed proteins of velvet beans grown in Nigeria. The results obtained were intended to provide a framework for thorough structural characterization that would, in turn, lead to the identification of specific protein species in *Mucuna* seed and a definition of their roles during seed development.

2. Materials and methods

2.1. Plant materials and chemicals

Trypsin, chymotrypsin, N-benzoyl-Phe-Val-Arg p-nitroanilide and N-benzoyl-Tyr p-nitroanilide were all purchased from Sigma (St Louis, USA). *Mucuna* seeds were obtained from Dr. Shirley Tarawali, International Livestock Research Institute, Ibadan sub-station, Nigeria.

2.2. Fractionation of albumin- and globulin-enriched fractions

The procedure for separation of seed proteins into albumin- and globulin-enriched fractions was modified from that described by Rudiger (1993). *Mucuna* seed meal was extracted in 0.05 M Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl, 1mM $CaCl_2$ and 1 mM $MgCl_2$ for 2 h at 4°C. The extract was filtered through miracloth and centrifuged at 23,500 *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 23,500 *g* for 20 min at 4°C. The precipitate obtained was re-suspended in distilled water and lyophilized. This served as the globulin-enriched fraction. The albumin-enriched supernatant solution was adjusted to pH 8.0 with 1 M NaOH, dialysed and lyophilised.

2.3. Total protein extraction and protein assays

The procedure for total protein fractionation was modified from Sun and Hall (1975). Tissues were homogenized with a Warring Blender prior to extraction in borate buffer (20 mM borate, 1 mN EDTA, pH 8.9) containing 0.5 M NaCl overnight, with stirring at 4°C. The extract was centrifuged at 20,000 *g* for 15 min at 4°C and the resultant supernatant dialysed for 24–48 h against several changes of distilled water. The dialysate was centrifuged again at 20,000 *g* for 15 min at 4°C. Supernatants obtained were then frozen, lyophilized and re-suspended in water at the appropriate concentration when needed. Protein concentration was estimated according to Bradford (1976) using bovine serum albumin as the standard.

2.4. Acrylamide gel electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using 12% acrylamide gels (Laemmli, 1970). When required, reduction was performed with 5% 2-mercaptoethanol (2-ME) added to the protein samples prior to loading. Gels were either stained with Coomassie Brilliant Blue R-250 or with Bio-Rad's Copper Stain and Destain reagent solution and calibrated using the mid-range protein molecular weight standards (Promega, Madison, USA).

2.5. Preparative native-PAGE

Preparative electrophoresis was carried out in the Model 491 Prep Cell (Bio-Rad Laboratories, CA, USA) using a 10-cm high acrylamide resolving gel (6%) and a 2-cm high stacking gel (3.75%). Prior to protein (100 mg) loading on top of the stacking gel, 2.5 ml of loading buffer (0.5 M Tris–HCl, pH 6.8, 0.5% bromophenol blue and 10% glycerol) was added to the sample. The same (continuous) buffer (50 mM Tris and 25 mM Borate, pH 8.7) was used in the electrode chamber and the gel. Electrophoresis was carried out at a constant 200 volts for 8 h at 4°C. Eluted samples were desalted through dialysis and lyophilized prior to use for N-terminal sequencing.

2.6. Protease inhibitor assays

The ability of *Mucuna* protease inhibitors to inhibit activities of trypsin and chymotrypsin was tested according to the procedure described by Saarikoski, Clapham and Von Arnold (1996). N-benzoyl-Phe-Val-Arg p-nitroanilide was used as a substrate for trypsin. For chymotrypsin, N-benzoyl-Tyr p-nitroanilide was used as a substrate. Total protein extracts acted as sources of inhibitor. Inhibition of the extract was determined by comparing the reduction in enzymatic activity on the addition of the extract with that given by the same enzyme concentration in the absence of any inhibitor. The same extract was used for both trypsin and chymotrypsin assays. All experiments were done in triplicate and repeated at least twice.

2.7. N-terminal sequencing

Polypeptide bands were separated by SDS–PAGE and electroblotted onto a polyvinylidene difluoride

(PVDF) membrane. The desired bands were then excised and sequenced on an Applied Biosystems model 477A ABI protein sequencer with a 120 A analyzer at the School of Biological Sciences, University of Durham (UK).

2.8. Developmental study

Developing seeds were collected from mature plants of *M. pruriens* var. *utilis* that were tagged at anthesis. Pods were harvested at one-week intervals from the time of flower fall, frozen in liquid nitrogen and stored at −80°C. The seeds from these pods were used as a source of total protein for Western blotting.

2.9. Production of rabbit antisera and immunoblotting

Polyclonal antisera was prepared by injecting rabbit with 1 mg of protein emulsified in Freund's complete adjuvant. Blood was collected from the ear vein of the rabbit 10 days after the booster injection. Serum was separated by centrifugation at 1000 *g* for 10 min. Protein transfer to a nitrocellulose membrane and immunoblotting was carried out according to procedures outlined in Bio-Rad technical manuals using the Mini Trans-Blot Transfer Cell system. The secondary antibody was goat anti-rabbit immunoglobulins coupled to alkaline phosphatase conjugate.

2.10. Haemagglutination activity

Haemagglutination assays using trypsinized rabbit erythrocytes were carried out on total protein extracts as previously described (Machuka, Okeola, Van Damme, Chrispeels, Van Leuven & Peumans, in press).

3. Results and discussion

3.1. Analysis of albumin and globulin fractions

Albumin- and globulin-enriched fractions were extracted from mature, dry seeds of seven varieties of *M. pruriens*, namely, Veracruz-White, Utilis, *IRZ*, Pruriensis, Cochinensis, Rajada and Ghana. Fig. 1 shows the SDS–PAGE profiles of the albumin and globulin fractions obtained under reducing conditions, i.e. in the presence of 2-ME. The most prominent polypeptide band profiles are indicated as shown in Fig. 1. In total, seven (designated a1–a7) abundant albumin and six (designated g1–g6) globulin polypeptide band patterns were seen after SDS–PAGE under reducing conditions. Other minor bands were also visualized in both fractions. In general, no obvious varietal differences were observed in the band patterns. However, clear-cut differences were observed in the number, size and intensity of bands when albumin and globulin fractions were compared. Within the albumin class, the most abundant polypeptides were a4 (21–30 kDa), followed by a2 (55–60 kDa) groups. The g3 (55–60 kDa) and g5 (22–31 kDa) groups constituted the most prominent cluster within the globulin class. Although most protocols for fractionation of albumins and globulins use phosphate buffer (Khan, Gatehouse & Boulter, 1980; Siddhuraju, Vijayakumari & Jarnardhanan, 1997), this buffer was avoided since phosphate ions may scavenge or precipitate metal ions that may be necessary for lectin activity. However. when it is used, the predominant albumin bands on SDS–PAGE gels were of low molecular weight (21–30 kDa), corresponding to the a4 group, whereas globulin profiles were similar to those described above (data not shown).

In order to detect the presence of proteins that contain disulfide linkages, SDS–PAGE profiles obtained in the presence of 2-ME were compared to profiles obtained in the absence of 2-ME (non-reducing conditions). As shown in Fig. 2, the polypeptide banding patterns under non-reducing conditions (Fig. 2, B, C) were different from those obtained under reducing conditions (Fig. 2, A, D). Since it is not possible to prove the corresponding patterns merely by comparing bands on SDS–PAGE gels performed with and without 2-ME, bands obtained using non-reducing conditions were further designated a8–a13 (albumins) and g7–g1 (globulins) (Fig. 2, B, C). As expected, the numbers of albumin and globulin bands were less when 2-ME was absent. For example, the low molecular weight bands a5, a6 and a7 did not appear on lone SDS–PAGE gels, indicating that they are likely to represent subunits of larger holoproteins. Similarly, although at least six globulin bands within the g5 cluster were visualized under reducing conditions, only three sharp bands were seen on SDS gels (Fig. 2,C, D). Since legume vicilins lack disulfide bonds (Casey, Domeney & Ellis, 1986), their electrophoretic pattern is likely to have remained unaffected by the use of 2-ME.

3.2. N-terminal protein sequencing

Preparative native-PAGE was used to separate three polypeptide bands (Fig. 3,d, e, f) present in the a4 albumin cluster (Fig. 1A). The N-terminal amino acid sequence of these bands, as well as a5, a6 and a7 (Figs 1A and 2A) was determined and is presented in Fig. 4. The sequences of a5 and a6 are identical to each other but different from a7, d and e. It is possible that these five bands reperesent homologous (a5 and a6) or heterologous (e.g. d and e) polypeptide subunits of the same protein, or that they represent a family of structurally-related proteins. Analysis of these sequences revealed similarities to the soybean Kunitz-type trypsin inhibitor, KTi12 (Jofuku & Goldberg, 1989). The consensus sequence was DDREPV-DT–PL, which is part of the

characteristic conserved signature of soybean Kunitz type trypsin inhibitors found at their N-terminus (Jofuku & Goldberg, 1989). The soybean Kunitz trypsin inhibitor family is one of numerous families of serine protease inhibitors comprising several proteins that include KTi1 and KTi12 (Koiwa, Bressa & Hasegawa, 1997). Protein database searches did not reveal any homology to band f (Fig. 4).

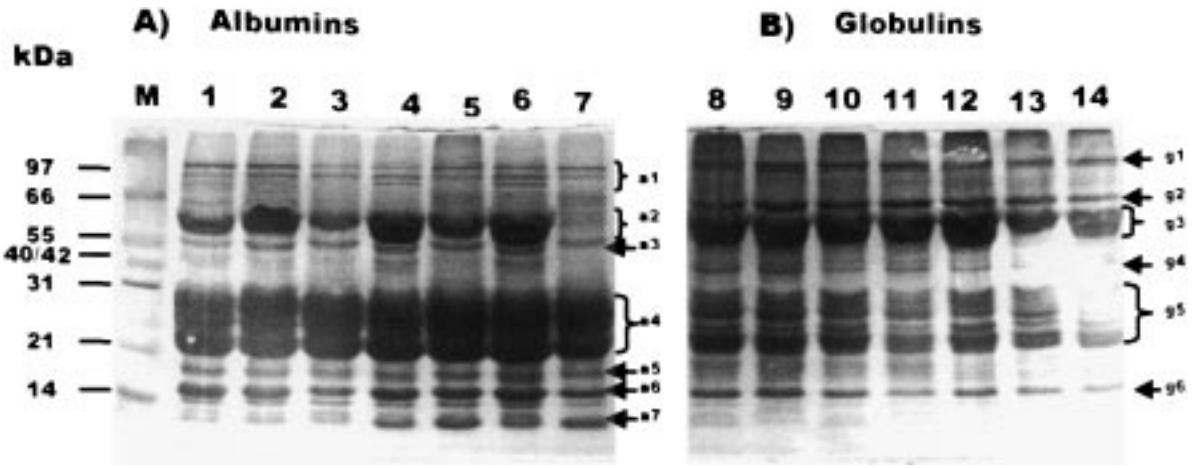

Fig. 1. SDS–PAGE (+2-ME) of albumin and globulin fractions of *Mucuna* seed proteins. Track 1 and 8, Rajada, 2 and 9, Ghana; 3 and 10, Utilis; 4 and 11, IR2; 5 and 12, Cochinensis; 6 and 13, Pruriensis; 7 and 14, *M*. VeraCruz-White. Prominent albumin (al–a7) and globulin (gl–g6) bands (arrows) and band clusters (right braces) are indicated. Molecular mass reference proteins in track M, in order of increasing molecular mass, are: lysozyme, soybean trypsin inhibitor, carbonic anhydrase, aldolase, ovalbumin, glutamate dehydrogenase, bovine serum albumin, and phosphorylase B.

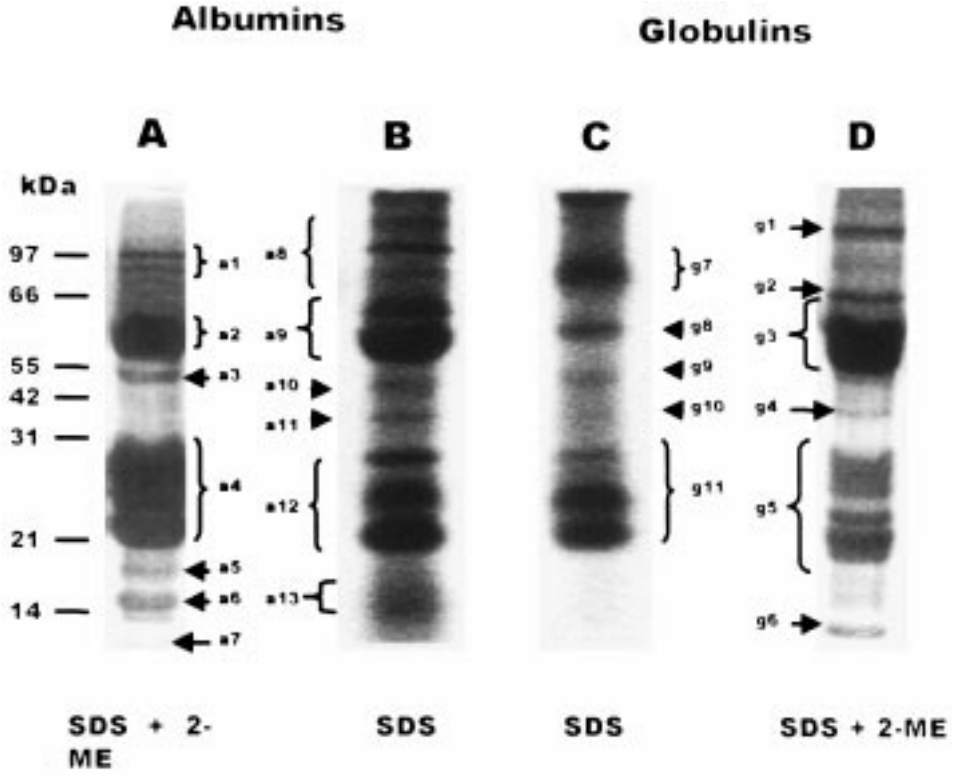

Fig. 2. SDS–PAGE of albumin and globulin fractions from *M. pruriens* var. Ghana under reducing (A and D) and non-reducing (B and C) conditions. Prominent albumin (a1–a13) and globulin (g1–g11) bands and band clusters are indicated with arrows and braces, respectively. Protein mid-range molecular weight reference markers indicated are as shown in the legend to Fig. 1.

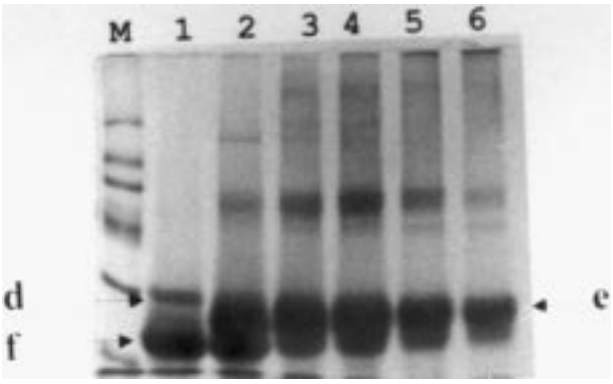

Fig. 3. Preparative native-PAGE of *M. pruriens* var. Ghana albumin fraction to separate the a4 cluster of bands shown in Figs 1 and 2. Numbers refer to the consecutive series of eluted fractions containing the three bands (d, e and f) which were separated (track 1 and 6) and used for N-terminal amino acid sequencing. Protein mid-range molecular weight reference markers in track M are as shown in the legend to Fig. 1.

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a5:      KNDGEPVRDTYGNPLL
a6:      KNDGEPVRDTYGNPLL
a7:      KDDREPVRDDGNPLL
d:       KDDREPVFDVGGNNPL
e:       KNDAEPVFDVGGNNPL
KTi12:   SDDREPVLDTDDDPLQ
f        PGVIFTGVTPLDDEEFKKPN
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Fig. 4. N-terminal amino acid sequences of albumin polypeptides (a5, a6, a7 in Fig. 2A and d, e and f in Fig. 3) from *M. pruriens* var. Utilis. The sequences are compared to soybean Kunitz-type trypsin inhibitor (KTi 12). The SwissProt Accession Number for KTi 12 is P25273 (Jofuku & Goldberg, 1989).

3.3. Protease inhibitor and haemagglutination assays and developmental regulation

The presence of trypsin and chymotrypsin inhibitors was confirmed through enzyme inhibition assays performed using total proteins from nine *Mucuna* varieties. Agglutination assays were performed on this fraction as well as on the albumin fraction which had been separated by a procedure that preserves the agglutinating activity of lectins. This class of carbohydrate-binding proteins has been well characterized in legume seeds (e.g. Van Damme, Peumans, Pusztai & Bardoz, 1998; Machuka et al., in press). The results of these assays are shown in Table 1. Varieties which exhibited the highest trypsin inhibition levels were Utilis and Cochinensis, whereas Veracruz (Molten and White) produced the lowest percentage inhibition levels. In contrast, the percentage inhibition of chymotrypsin was very low ($< 18\%$) in all varieties. No agglutination was observed in all assays utilizing trypsinized rabbit erythrocytes, indicating low or undetectable lectin levels in the 11

Table 1
Trypsin, chymotrypsin inhibitor and agglutination activities of *Mucuna* protein extracts

Variety	% Inhibition		Agglutination[a]
	Trypsin	Chymotrypsin	
Rajada	53	10	None
Cochinensis	83	17	None
Ghana	69	15	None
Georgia	64	6	None
Preta	56	2	None
Jaspadea	58	2	None
VeraCruz-Black	75	17	None
VeraCruz-Mottle	23	8	None
VeraCruz-White	33	10	None
Utilis	82	2	None
IRZ	73	2	None

[a] Agglutination assays were performed using rabbit erythrocytes.

Mucuna varieties. Alternatively, the lectins may not be agglutinated by rabbit blood, but may be agglutinated by sheep, human or other kind of erythrocytes. Brazilian *M. pruriens* seeds lack agglutinating activity, not only against rabbit, but also pig and human erythrocytes (Udeliba & Carlini, 1998). The characterization of an N-acetylgalactosamine-specific lectin from *M. derringiana* by a combination of affinity chromatography and reversed phase HPLC by Mo and Goldstein (1994) demonstrates that at least some species of *Mucuna* contain extractable lectins in their seeds. However, the lectins appear to occur at naturally low concentrations. Moreover, their levels and concentrations vary with ecological and climatological conditions, as reported on Nigerian- and Brazilian-grown *M. pruriensis* var. Utilis by Udeliba and Carlini (1998).

The expression of one polypeptide (band e in Fig. 3) was followed during seed development through Western blotting using antibodies raised against this protein (Fig. 5). The analysis showed that this representative band is undetectable at anthesis but accumulates during seed development to reach peak levels when the seed attains full maturity. This observation provides evidence that this protein is a seed storage protein.

Using in situ hybridization, Perez-Grau and Goldberg (1989) reported a similar pattern in the accumulation soybean KTi1/2 mRNA at various stages of embryogenesis seed maturation. The accumulation of protease inhibitors in developing seeds, fruits and tubers, as well as inducibility by wounding, has been seen as an indication of their role, not only as storage proteins, but also as plant defence proteins (Giri, Harsulkar, Deshpande, Sainani, Gupta & Ranjekar, 1998; Koiwa et al., 1997; Reek et al., 1997). Judging from the pattern of accumulation of the abundant *Mucuna* seed protein species (Fig. 5A), it is likely that most of them have a storage role (Bewley & Black, 1994). Low concentrations of lectins in Nigerian *Mucuna* seed may mean that they

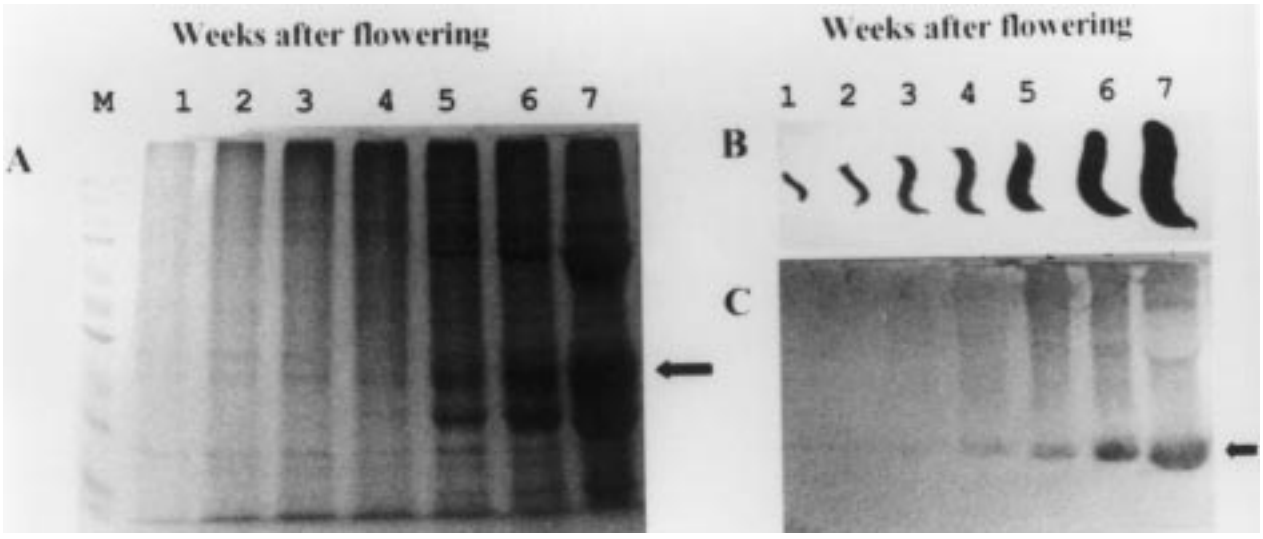

Fig. 5. Western blotting of total protein (A) from developing seeds (B) of *M. pruriens* var. Utilis with antibody raised against a polypeptide (Fig. 3, band e) from the albumin fraction (C). The antibody was raised as described in Materials and Methods. Numbers 1–7 represent weeks after flower fall. Approximately 30 μg of total protein was loaded in each well. Protein mid-range molecular weight reference markers in track M are as shown in the legend to Fig. 1.

do not play a major role as antinutritional factors in this under-utilized crop, unlike protease inhibitors which occur in high concentrations. Although Indian *Mucuna* seed protease inhibitors are inactivated by heating treatments, lectins could not be inactivated by autoclaving for 15 min (Siddhuraju et al., 1996). This suggests a requirement for long heating treatments when considering utilization of velvet beans as human and animal feed, as is the practice in West Africa (Lorenzetti et al., 1998).

The results presented in this paper provide a basis for further biochemical and molecular characterization of *Mucuna* seed proteins. Current investigations in this direction involve the purification of trypsin and chymotrypsin inhibitors by affinity chromatography and studies of their interaction with legume pod borer (*Maruca vitrata*) proteases and in artificial diet feeding assays (J. Machuka and L.E.N. Jackai, manuscript in preparation). Cloning of the genes encoding interesting protein species is also anticipated.

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