Bot. Bull. Acad. Sin. (2005) **46:** 189-196

Characterization of Alpha-amylase inhibitor in *Vigna sublobata*

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(Received August 30, 2004; Accepted January 14, 2005)

Abstract. Alpha-amylase inhibitor protein, which inhibits the activity of insect (*Callosobruchus analis*) α-amylase, was characterized from *V. sublobata*. The molecular weight of purified inhibitor protein was 14 kDa by SDS-PAGE. The inhibitor is non-glycosylated protein and its N-terminal sequence is similar (A P S P V…) to *Phaseolus vulgaris* α-AI-1. Its pI value is 6.0 and largely localised in cotyledons. The inhibitory activity decreased during germination from days one to five. In the developmental stages of seed formation from anthesis to 30 days the inhibitor content increased.

Keywords: α-Amylase; Alpha-amylase inhibitor; *Callosobruchus analis*; *Vigna sublobata*.

Introduction

Many edible plant seeds contain substances that inhibit enzymes, especially hydrolases. Most of these compounds are proteins by nature, which specifically inhibit enzymes by forming complexes that block the active site or alter enzyme conformation, ultimately reducing the catalytic function. Substances present in the seeds of *P. vulgaris* suggested to play a role in insect resistance include heteropolysaccharides, lectins, and protease- and amylase inhibitors (Applebaum et al., 1969). It has also been suggested that the various forms of resistance are mainly due to the presence of genes of the lectin family (Chrispeels and Raikhel, 1991).

Among the storage pests, the genus- *Callosobruchus* (Coleoptera: Bruchidae) is the most serious pest on mungbean and other *Vigna* species (Talekar and Lin, 1981). Alpha-amylase inhibitors have been studied in several common beans (Marshall and Lauda, 1975) maize (Blanco-Labra and Iturbe-Chinas, 1981), sorghum (Kutty and Pattabiraman, 1986), wheat (Warchalewski, 1977), and barley (Mundy and Rogers, 1986). Seed alpha-amylase inhibitor (α-AI) in several cultivars of the common bean plays a protective role against bruchid pests (Ishimoto and Kitamura, 1989). The α-AI strongly inhibited the larval midgut α-amylase activities of adzuki bean weevil (*C. chinensis* L.) and cowpea weevil (*C. maculatus*), non-pest species of the common bean. The gene encoding an α-AI was isolated from common bean and transferred to pea (*Pisum sativum*), and transgenic peas were produced that expressed the transgene (Shade et al., 1994). Bean α-AI-1 in transgenic peas provided complete protection from pea weevil (*Bruchus pisorum*) under field conditions (Morton et al., 2000). Earlier studies from our laboratory also reported that the presence of α-AI is one among the possible strong factors for bruchid resistance in the wild relatives of mungbean, *V. sublobata* (Sahu, 1996). In the present study an α-AI from *V. sublobata* has been isolated from seed and characterized.

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Materials and Methods

Source of Seed Materials

Seeds of *Vigna* genotypes, including viz., green gram (var. CO 4, CO 5, K-851, P-9078), black gram (var. CO 5), *V. glabracens*, *V. sublobata*, *V. trilobata* and *V. umbellata* obtained from the Department of Pulses, Center for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore were used.

Extraction of Seed Proteins

The extraction of proteins from seed flour was done following the method of Mirkov et al. (1994) for electrophoresis (SDS-PAGE) and immunological analysis. One hundred mg of finely ground seed flour was extracted in the extraction buffer (10 mM Tris-HCl [pH 7.5], 500 mM NaCl, 1% 2-mercapto-ethanol, 0.1% triton-X-100, 2 mM phenylmethylsulphonyl fluoride [PMSF] [1 ml/mg sample]) by homogenization followed by incubation at 4°C for 1 h. It was then centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was collected and stored frozen in aliquots. The protein content in the extract was quantified following Bradford's (1976) method.

α-AI Assay

The α-AI was assayed by quantifying the reducing sugar (maltose equivalent) liberated under the assay conditions. The enzyme inhibitory activity is expressed as the decrease in units of maltose liberated. A modified dinitrosalicylic acid (DNS) method of Bernfeld (1955) was

followed to estimate the maltose content. Two hundred microlitres of α-AI extract was pre-incubated with 50 µl of the larval amylase for 30 min, and 1 ml (1% w/v) starch solution was added before further incubating at 37°C for 10 min. The reaction was stopped by adding 1 ml DNS reagent (1 g DNS, 200 mg crystalline phenol, and 50 mg sodium sulphite dissolved in 1% NaOH [w/v] [prepared fresh]), and then the contents were heated in a boiling-water bath for 5 min. One ml potassium sodium tartrate (40% [w/v]) solution was added while the contents were still warm and volume in each tube was made up to 10 ml. A blank was set without α-AI extract, and another one without amylase enzyme, replaced by equal quantities of extraction buffer. The absorbance was measured at 530 nm. The reducing sugar released from starch was estimated as maltose (1 mg/ml) equivalent from the standard graph.

Purification of α-AI

Finely ground seed flour (250 g) of *V. sublobata* was extracted in 1l of extraction buffer (10 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1% 2-mercapto-ethanol, 0.1% Triton-X-100, 2 mM phenylmethylsulphonyl fluoride, PMSF). The crude protein extract was subjected to ammonium sulphate precipitation (80-100% saturation) at room temperature. The precipitate dissolved in extraction buffer and was dialyzed against water in batches. The dialyzed material was lyophilized and stored at -20°C until further analysis.

The α-AI was fractionated by repeated size exclusion chromatography on a Sephadex G-50 column (26 × 1.2 cm) with 20 mM phosphate buffer (pH 7.0) and then by reversed phased high performance liquid chromatography (RP-HPLC) on C_{18} column (5 µ column 250 × 4.6 mm). Mobile phase used was acetonitrile : water (70:30) containing 0.1% trifluoroacetic acid (TFA).

Extraction of Alpha-Amylase from Larvae of C. analis

The procedure adopted is a modification of the method used by Bernfeld et al. (1955). Infested greengram seeds were dissected, and the active larvae (100 no.) were homogenized in 2 ml extraction buffer (20 mM sodium phosphate [pH 6.9], 300 mM sodiumchloride, 2 mM calcium chloride) followed by centrifugation at 15,000 rpm for 30 min at 4°C. The supernatant was distributed into 0.5 ml aliquots and stored at -20°C. This was used as the enzyme extract without any dilution.

Electrophoresis (SDS-PAGE) of Proteins and Immunoblotting

The polypeptides in the samples were fractionated in (15%) polyacrylamide gel in a discontinuous buffer system as described by Laemmli (1970). Polypeptide markers (medium range) were used to estimate the molecular weight of resolved polypeptides. Western blotting and immunostaining of α-AI were carried out as described (Bio-Rad technical bulletin). Pea α-AI antiserum (gift from Dr. Willy Peumans, Belgium) was used.

N-Terminal Amino Acid Sequencing

The N-terminal partial amino acid sequence of the purified α-AI of *V. sublobata* was determined by Edman degradation using the National Facility available at the Department of Biochemistry, Indian Institute of Science, Bangalore.

Results and Discussion

Among the genotypes screened by conducting preference and non-preference studies of the bruchid pest, *C. analis*, *Vigna sublobata*, and *V. umbellate,* showed resistance to the bruchid attack. Crude protein extracts from the seeds of ten *Vigna* genotypes were assayed for inhibitory activity against the larval α-amylase of *C. analis*. *Vigna umbellata, V. sublobata*, and *V. glabracens* showed higher levels of inhibitory activity. *Vigna trilobata* showed a moderate level of inhibition while other *Vigna* species showed lower inhibitory activity (Table 1). Intervarietal variation in the content of α-AIs in seeds is not uncommon and has been reported in crops such as common bean (Ishimoto and Kitamura, 1991; Ishimoto et al., 1995; Baker et al., 1991; Sanchez-Monge et al., 1996), cowpea (Prasad et al., 1996), barley (Jarrett et al., 1997), and rye (Taeufel et al., 1997). The α-AI content in chickpea varieties ranged from 11.6 to 84.4 units/g seed (Mullimani et al., 1994).

Table 1. Seed alpha-amylase inhibitor activity of different *Vigna species* on bruchid α-amylase.

Genotype	Protein content* (mg/g seed)	Inhibitory activity % against α-amylase of *C. analis**
Green gram (*Vigna radiata*) var. C04	13.1	11.41
C05	12.6	10.65
K-851	13.5	11.08
P-9078	13.9	12.50
Black gram (*V. radiata*) var. C05	15.1	12.01
Rice bean (*V. umbellata*)	14.5	110.01
V. sublobata	13.5	80.50
V. trilobata	13.9	27.45
V. glabracens	14.1	70.03
V. unguiculata	14.3	20.05
Critical Difference (0.05)	1.41	4.99

*Mean of three experiments.

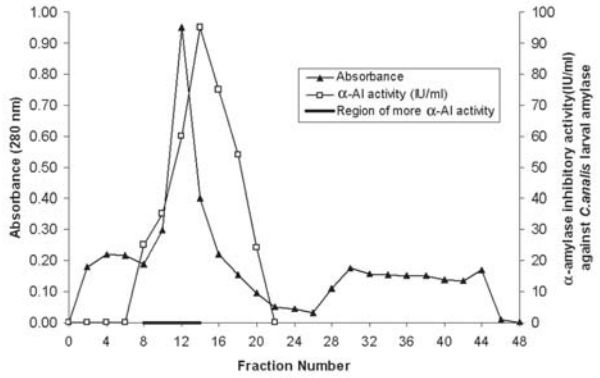

Figure 1. Gel exclusion chromatograph of *V. sublobata* α-AI on Sephadex G-50 column. Lyophilized sample (2 mg/ml) in equilibration buffer (0.02 M sodium phosphate, pH 6.9 containing 0.3 M NaCl) was applied on to Sephadex G-50 column (26 × 1.2 cm) bed volume 30 ml.

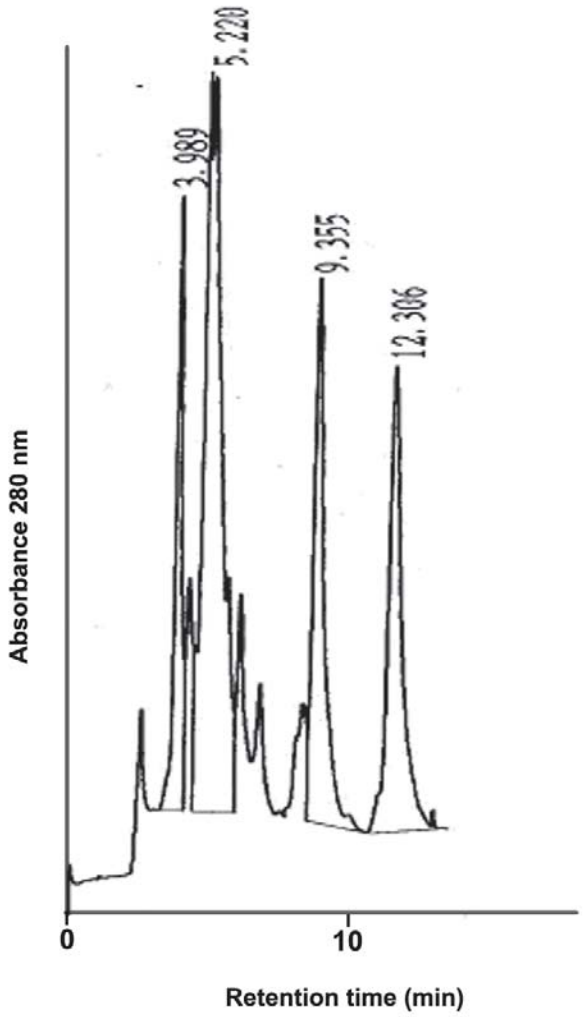

Figure 2. HPLC profile of *V. sublobata* crude protein extract from seed. The fraction obtained from Sephadex G-50 column was subjected to RP-HPLC (Hypersil BDS C18, 5 µ column [250 × 4.6 mm]). Fifty microlitre of fraction was injected into the system. The proteins were eluted using a linear gradient of 70% acetonitrile containing 0.1% TFA.

Purification of α-AI

Crude protein extract of *V. sublobata* seeds was precipitated using ammonium sulphate at different levels of saturation. Greater α-amylase inhibitory activity (85%) was observed in the fraction precipitated between 80 and 100 percent ammonium sulphate saturation. This fraction was re-suspended in phosphate buffer (pH 7.0) and subsequently applied onto a Sephadex G-50 column. The chromotograph of *V. sublobata* (Figure 1) showed a minor peak followed by a major peak. The fractions of major peak (fractions 10-14; 25 ml) that showed inhibitory activity were pooled.

RP-HPLC

Sephadex G-50 column-eluted fraction was further fractionated on a C_{18} column (BDS, Shimadzu). The profile showed six major peaks and five minor peaks (Figure 2). Assay of major peaks (Table 2) revealed that the only peak with retention time 5.2 min of *V. sublobata* strongly inhibited the *C. analis* larval α-amylase while others did not.

The protein content of *V. sublobata* was estimated at 13.5 mg/g seed. For the α-AI purification process 3375 mg crude protein was extracted from 250 g seed flour. Based on the colorimetric assay, it was found that the specific activity of the inhibitor at this stage was very low. In the present study, the seeds of *V. sublobata* contained 0.8-1. 0 percent of the α-AI and showed resistance to bruchids. Subsequent chromatographic separation including RP-HPLC removed another small percentage of the total proteins. The procedure used to purify this inhibitor gave a pure protein, the characteristics of which were closely related to other inhibitors previously reported in different beans.

Ho and Whitaker (1993) achieved an 18.5-fold increase in the specific activity of white kidney bean α-AI from the aqueous crude extract by heat denaturation, ethanol fractionation, and subsequent chromatographic separations on DEAE cellulose column. Transgenic pea seeds, which completely resisted bruchid attack, contained 1-2 percent of the α-AI (Marshall and Lauda, 1975; Blanco-Labra and Iturbe-Chinas, 1981). Fold purification of alpha-amylase inhibitor from *V. sublobata* is summarized in Table 3.

Molecular Weight

The purified α-AI fraction from *V. sublobata* showed a molecular weight of 14 kDa (approximately) as estimated

Table 2. Seed alpha-amylase inhibitor activity of *V. sublobata* from different C_{18} column fractions on larval midgut α-amylase of *C. analis*.

Peak No.	Retention time (min)	Protein content (µg/ml)	Inhibitory activity (IU/ml)
1	5.214	260.0	250.49
2	5.899	180.0	0.00
3	6.573	200.5	0.00

Table 3. Purification of alpha-amylase inhibitor from *V. sublobata*.

Activity (IU/ml)	Protein content (mg/ml)	Specific activity (IU/mg protein)	Recovery activity (%)	Fold purification
23.1	1.642	14.06	100	1.00
691	8.629	80.07	72.9	4.4
438.2	3.241	135.20	63.7	7.48
250.49	0.271	924.31	3.8	55.17

by Sephadex G-50 gel filtration in relation to standard proteins (aprotinin, cytochrome-C, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase b) chromatographed, separately. The analysis of the purified protein on SDS-PAGE confirmed the above observation. The α-AI showed a molecular weight of 14 kDa on a 15% PAGE containing SDS (Figure 3). Ho and Whitaker (1993) found that the molecular weight of white kidney bean α-AI (WKB 858 A) was 42, 40 and 38 kDa on FPLC superose gel filtration chromatography, Sephadex G-75 gel filtration chromatography, and native gel electrophoresis, respectively. α-AIs form a large family of proteins with varying molecular masses. Insect-specific albumins are low molecular weight proteins of 12-24 kDa (Feng et al., 1996). Rice α-AIs are relatively small proteins of around 14 kDa (Feng et al., 1991).

Native Gel Analysis

The purified α-AI protein from *V. sublobata* was subjected to electrophoresis under non-denaturing and non-reducing conditions. The results showed a single band after silver staining revealing the (near) homogeneity of the purified fraction (Figure 4).

Western Blot Patterns

Western blot analysis of *V. sublobata* using French bean α-AI antiserum revealed an array of at least five bands in the low molecular weight region. The weak signals seen in the high molecular weight range may be due to other proteins, such as arcelin and phytohaemagglutinins (PHA), antigenically related to α-AI. The Western blot analysis of purified proteins from *V. sublobata* using French bean α-AI antiserum clearly showed a single band in the 14 kDa region (Figure 5). The French bean α-AI antiserum used in the present study is polyclonal in nature and hence capable of producing many immunosignals. The proteins from other crop seeds have also been reported to have molecular weights in the range of 14-20 kDa. In addition, many different types of α-AI do occur. The result of the present study is in accordance with that of the report by

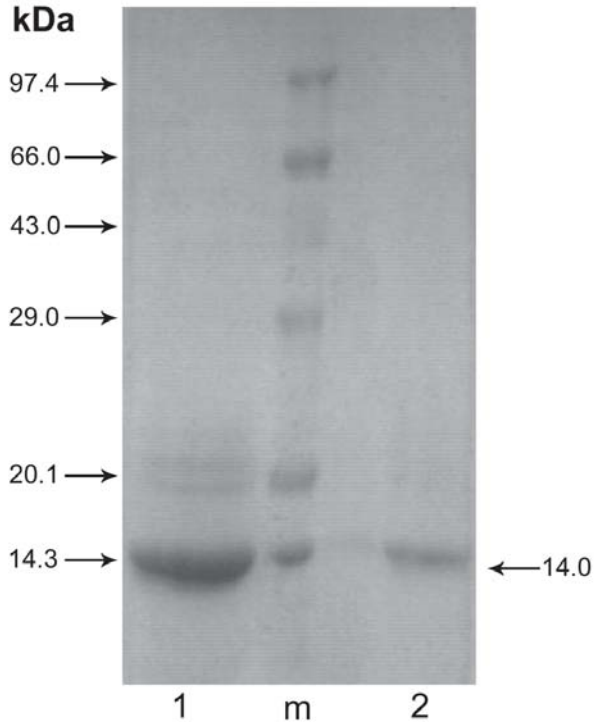

Figure 3. Molecular weight of α-AI from *V. sublobata* seed. Crude proteins (≈20 mg) were fractionated on 15% SDS-PAGE. Lane 1, 80-100% ammonium sulphate precipitated proteins; lane 2, Sephadex G-50 column eluted protein; and lane m, molecular weight marker.

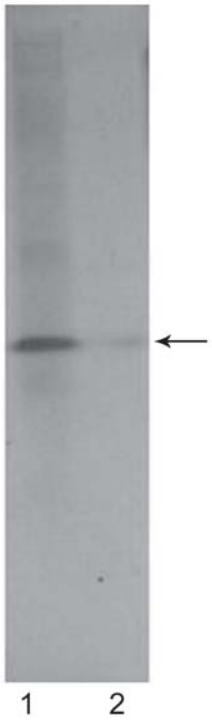

Figure 4. Purified α-AI from *V. sublobata* seed on a native gel. Protein (»5 mg) was electrophoresed on 15% gel under non-reducing and non-denaturing conditions and silver stained. Lane 1, crude extract and lane 2, purified protein.

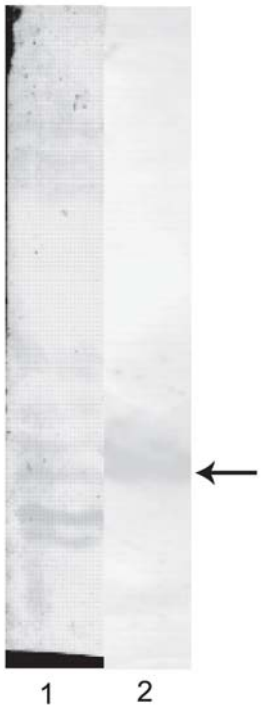

Figure 5. Protein blot analysis. Immunoblot of SDS-PAGE separated polypeptides from *V. sublobata* (lane 1); purified protein (5 μg) on immunoblot was probed with French bean α-AI antiserum (lane 2).

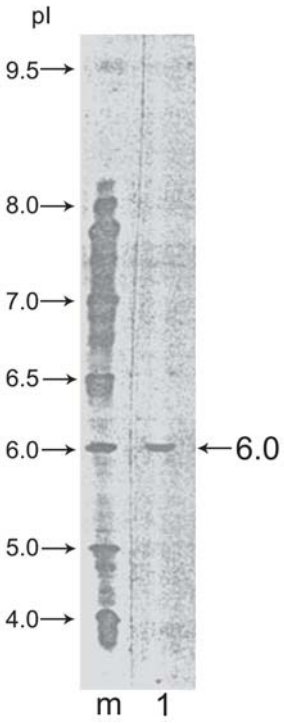

Figure 6. Isoelectric focusing of the purified α-AI. Five μg of the purified α-AI protein from *V. sublobata* was subjected to isoelectric focusing. Lane m, pI markers; lane 1, *V. sublobata*.

Iguti and Lajolo (1991), wherein isoforms of α-AI have been reported in French bean.

N-Terminal Sequence

The purified protein of *V. sublobata* was transferred onto PVDF membrane and subjected to sequencing. The first five amino acids in the N-terminus are: Ala-Pro-Ser-Pro-Val (A P S P V). Furthermore, the protein is not N-terminally blocked. The first five amino acid residues of *V. sublobata* α-AI are similar to those of *P. vulgaris* α-AI-1 (Blanco-Labra et al., 1996). The N-terminus of *V. sublobata* inhibitor showed less homology with the already characterized legume and cereal α-AIs. French bean α-AI antiserum cross-reacted with *V. sublobata* has an amino acid sequence more or less similar to *P. vulgaris* α-AI-1.

pI of the Protein

The α-AI protein from *V. sublobata* was focused at pI 6.0 (Figure 6). Common bean α-AI-1 showed wider pI values (4.5-6.5). The pI value for tepary bean is 4.7 (Blanco-Labra et al., 1996). Most of the reported α-AI proteins showed pI between 4.5 and 9.0. Blanco Labra et al. (1996) reported the α-AI of tepary bean (*Phaseolus acutifolius*) to be pH 4.7.

Localisation of α-AI

The protein is mainly localized in the cotyledons and embryonic axes as examined by SDS-PAGE followed by silver staining (Figure 7). Dot blot analysis with French bean

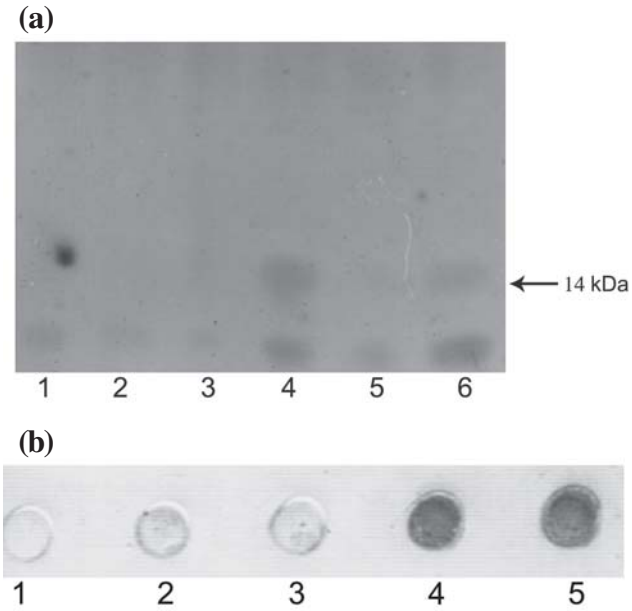

Figure 7. (a) Localization of α-AI protein from *V. sublobata*. Protein (5 μg) were fractionated on 12% SDS-PAGE and silver-stained. Lane 1, leaf; lane 2, stem; lane 3, seed coat; lane 4, cotyledon; lane 5, root and lane 6, embryonic axis. (b) Dot blot analysis for the localization of α-AI in different tissues of *V. sublobata*. Crude extract (20 mg protein) were probed with French bean α-AI antiserum. Dot 1, seed coat; dot 2, leaf; dot 3, stem; dot 4, cotyledon and dot 5, embryonic axis.

α-AI antiserum reinforced the above observation (Figure 7). Earlier studies by Moreno et al. (1989) showed that in common bean the α-AI was detected only in the cotyledons and embryonic axis.

α-AI in V. sublobata During Germination and Seed Development

The fate of α-AI during seed germination was followed by assaying the inhibition of *C. anlis* amylase using the extracts prepared at 24-h intervals from the time of soaking to the 10th day of germination. The inhibitory activity decreased from day one through five. No activity was observed during subsequent days of germination (Table 4). SDS-PAGE also showed that the α-AI (14 kDa) polypeptide band disappeared beyond 8 days of germination (Data not shown).

During seed development, the inhibitor accumulated in the seeds at levels detectable by ELISA within 15 days of seed development (Table 5). The inhibitory activity was nearly or completely undetectable by both colorimetric and ELISA until day 10 of seed development. The level increased up to 30 days of seed formation. Thereafter, the inhibitor level remained unaltered or slightly decreased during the drying of the seed.

As the germination progressed, the seed inhibitor content decreased. The inhibitor was undetectable in the cotyledons by day 5 of germination (Table 3). This result is in accordance with the findings of Baumgartner and Chrispeels (1976) in mungbean. Moreno et al. (1989) showed that in bean seeds the α-AI appeared in detectable amounts seventeen days. The characterisation of new inhibitors, such as the one isolated in the present work, is important considering that inhibitors obtained from different sources differ in characteristics. In this work the *V. sublobata* α-AI demonstrated activity against *C. analis* that attack mungbean (*V. radiata*). Considering that *V. sublobata* can hybridize with *V. radiata* and *V. mungo*, improving this crop may be possible through traditional breeding programs, with the aim of obtaining materials of better specific resistance characteristics to these insect pests.

Table 4. Decline of in α-AI level in *V. sublobata* seed during germination.

Germination period (days)	Protein content (mg/g) seed*	Inhibition on larval midgut α-amylase (IU/g sample)*
0	13.50	104.6
1	13.45	120.9
2	12.00	90.1
3	11.50	45.5
4	11.06	25.6
5	10.93	0.0
6	10.84	0.0
7	10.72	0.0
8	10.43	0.0
9	10.22	0.0
10	10.09	0.0
Critical difference (0.05)	0.59	2.04

*Mean of two experiments.

Table 5. Accumulation of *V. sublobata* α-AI during seed development.

Stage (days after anthesis)	Protein content (mg/g fresh seed)*	α-AI level (against larval midgut α-amylase of *C. analis*) IU/g seed*	
		Colorimetric	ELISA (A 405/g seed)
0	7.102	0.00	0.00
5	6.405	0.00	0.00
10	6.302	0.00	0.00
15	6.766	45.23	10.21
20	6.823	93.37	15.35
25	9.478	126.25	20.02
30	10.405	151.39	32.32
Dry seed	13.500	140.60	26.75
One month after storage	14.000	145.20	39.75
Critical difference (0.05)	1.33	2.58	1.53

*Mean of two experiments.

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Vigna sublobata 之 α-amylase（α-澱粉水解酶）抑制劑之性狀

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本文描述來自*Vigna sublobata* 之α-澱粉水解酶的抑制蛋白的性狀，該蛋白可抑制昆蟲（*Callosobruchus analis*）腸道之 α-水解酶。SDS-電泳分析顯示該純化之抑制蛋白分子量為 14,000。該抑制蛋白係非醣化者；而 N-端序列（A P S P V...）類似來自 *Phaseolus vulgaris* 之 α-AI-1，它的 PI 值為 6.0。它主要位於子葉。當種子發芽後第一天到第五天該抑制蛋白之活性逐漸下降。而當種子發育形成階段（即授粉後直到第三十天）該抑制蛋白含量逐漸增加。

關鍵詞：α-澱粉水解酶；*Callosobruchus analis*；*Vigna sublobata*。