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Purification and characterization of *Vigna unguiculata* cultivar asparaginase

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Asparaginase (EC 3.5.1.1) activity was determined in non-germinating and germinating seeds of five Egyptian cowpea (*Vigna unguiculata*) cultivars (Kareem 7, Dokki 331, Kafer El-Sheikh 1, Kaha 1, and Fodder). In all cultivars, asparaginase specific activity was higher in germinating seeds. The highest specific activity of asparaginase was observed in Fodder cultivar. Asparaginase was purified from Fodder cultivar germinating seeds and resolved, by DEAE sepharose, into three forms named ASPG I, ASPG II, and ASPG III. The molecular mass of ASPG II was 70 kDa for native enzyme using gel filtration and ~35 kDa after SDS-PAGE electrophoresis. This indicates a dimeric structure for ASPG II. ASPG II had a Km value of 1.25 mM for asparagines, a pH optimum at 8.0 and temperature optimum and heat stability at 40˚C. The Fodder cultivar ASPG II was specific for L-asparagine, did not hydrolyze D-asparagine and was not specific for L-glutamine. Ni^{2+} and Co^{2+} may act as activators on ASPG II but Hg^{2+}, Ba^{2+}, Zn^{2+} and Mn^{2+} had inhibitory effect. The purified asparaginase may be valuable in chemotherapeutic treatments.

Key words: asparagine, glutamine, seed nitrogen supply, cowpea, legume species.

INTRODUCTION

L-asparaginases (EC 3.5.1.1) are enzymes that catalyze the hydrolysis of the amide group of L-asparagine, releasing L-aspartate and ammonia. Plant L-asparaginases belong to the superfamily of N-terminal nucleophile (Ntn) amidohydrolases. Based on amino acid sequencing and biochemical properties, the enzymes with asparaginase can be divided into several families. The two largest and best-characterized families include bacterial and plant type asparaginases (Borek & Jaskolski, 2001; Borek *et al.*, 2004; Michalaska *et al.*, 2006). The reaction of L-asparagine hydrolysis in plants is catalyzed by asparaginases, that have no homology to bacterial enzymes (Borek *et al.*, 1999). Enzymes related to plant asparaginases are also found in other organisms. In particular, three enzymes with asparaginase activity have been characterized in *Escherichia coli* (Borek *et al.*, 2004). EcAIII asparaginase shows catalytic activity and structure similarity to plant asparaginase (Borek *et al.*, 2004).

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The isolation of plant asparaginase has been less studied. In plants, L-asparagine is the major nitrogen storage and transport compound that is utilized in protein synthesis in actively growing tissues. Asparagine is the principle amino acid transported in xylem of both nodulated and non nodulated legume plants (Lima & Sodek, 2003). Asparagine accounts for approximately 50% or more of the total free amino acids in the developing soybean embryo at mid-maturation. Asparagine levels in the developing soybean embryo are related to seed protein content at maturity, suggesting a strict control of asparagines biosynthesis and catabolism in this tissue (Hernandez-Sebastia *et al.*, 2005).

In developing leaves of *Lupinus albus* asparagines utilization was shown to occur initially by deamidation and later in leaf development by transamination. The presence of asparaginase has been confirmed in various lupin species by a number of studies (Lea *et al.*, 1984; Lough *et al.*, 1992), and asparaginase activity has also been reported in *Glycine max* (Streeter, 1977) and *Pisum sativum* (Murray & Kennedy, 1980; Sodek *et al.*, 1980).

L-asparaginase is a therapeutically important protein used in combination with other drugs in the treatment of acute lymphocytic leukemia (mainly in children), Hodgkin's disease, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma, reticlesarcoma and melanosarcoma (Stecher *et al.*, 1999; Verma *et al.*, 2007).

The aim of the present study is to identify and select among five Egyptian cowpea (*Vigna unguiculata*) cultivars the one showing the highest asparaginase activity and eventually to purify and characterize the selected asparaginase.

MATERIALS AND METHODS

Materials

Five Egyptian cowpea (*Vigna unguiculata* (L.) Walp.) cultivars (Kareem 7, Dokki 331, Kafer El-Sheikh 1, Kaha 1 and Fodder) were used in this study. The seeds were obtained from Agriculture Research Centre (Giza, Egypt). Asparagine was purchased from Sigma-Aldrich (St. Louis, Mo, USA). DEAE sepharose and Sephacryl S-200 were supplied from Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents used were of the highest grade available.

Germination of Vigna seeds

All seeds were surface sterilized with 0.25% (v/v) sodium hypochlorite solution for 5 min and washed 7 times with sterile water. The seeds were germinated on a piece of watered cotton in Petri dishes, and incubated at room temperature in the dark. Germinated seeds were harvested after 7 days when seedling had developed to a stage at which coleoptiles had expanded to 5-7 cm (Coombe *et al.*, 1967). The germinated seeds were stored at –20°C.

Preparation of seeds and germinating seeds extracts

Germinating and no-germinated seeds were separately homogenized in 50 mM potassium phosphate buffer, pH 8.0, containing 150 mM sodium chloride, 5 mM PMSF, 1 mM EDTA, and 10% (w/v) glycerol using a Teflon pestle homogenizer for 10 min. The homogenate was centrifuged at 13,200 *g* for 20 min to remove insoluble debris and the supernatant was designated as seed extract (Chang & Farnden, 1981).

Enzyme and protein assays

Asparaginase activity was measured in terms of rate of hydrolysis of L-asparagine by measuring the amount of ammonia released in the reaction. Reaction mixture in 1 ml contained 50 mM sodium phosphate buffer (pH 8.0), 2 mM EDTA, 8 mM L-asparagine and 0.2-1.2 units of enzyme. The reaction mixture was incubated at 37°C for 10 min and the reaction was terminated by adding 100 µl of 20% TCA. The mixture was centrifuged for 10 minutes at 3000 *g* and the amount of ammonia released was determined by Nessler's reagent (Barrett, 1935). 0.7 ml distilled water was added to 0.2 ml aliquot of supernatant and then 0.1 ml of Nessler's reagent was added. The absorbance was recorded at 480 nm after 10 min. Absorbance values were converted to µmoles of ammonia from the standard curve prepared with ammonium sulfate. One unit of enzyme activity was defined as the amount of enzyme required to release one µmole of ammonia per hour under standard assay conditions (Wriston, 1985). Protein concentration was determined using Bradford dye method with bovine serum albumin as a standard (Bradford, 1976). Three independent extracts from each cultivar of non-germinating and germinating seeds were performed from which the mean and standard errors of the specific activity of asparaginase were calculated.

Purification of asparaginase from V. unguiculata cv. Fodder

Enzyme isolation and purification were performed at 4-8°C.

The homogenate sample (2 g of germinating seeds) was dialyzed against 50 mM potassium phosphate buffer, pH 8.0 for 4 hrs and then it was applied directly to a DEAE spherose column (3.5×1.6 cm i.d.) equilibrated with 50 mM sodium phosphate buffer, pH 7.5. The enzymes were eluted with a step wise different concentration of NaCl; 0.0, 0.1, 0.2, 0.3 and 0.4 M NaCl prepared in the same buffer. Fractions were collected at a flow rate of 30 ml hr^{-1}. The eluted fractions were monitored at 280 nm for protein assayed (Warburg & Christian, 1942) and assayed for asparaginase activity. Three parts of protein fractions exhibiting asparaginase activity were pooled from three peeks (ASPG I to ASPG III) according to their elution order. ASPG II (which showed the highest asparaginase activity, see Results section) was concentrated by using dialysis bag with cut off 13,000 against sucrose. The concentrated sample was applied on Sephacryl S-200 column (90×1.6 cm i.d.) equilibrated with the 50 mM sodium phospate buffer pH 7.5. Fractions were collected at a flow rate of 20 ml hr^{-1}.

The eluted fractions were monitored for protein and assayed for enzyme activity. Molecular weight of proteins was determined by gel filtration technique using Sephacryl S-200. The column was calibrated with cytochrome c (12,400), carbonic anhydrase (29,000), bovine serum albumin (66,700), alcohol dehydrogenase (150,000) and β-amylase (200,000). Dextran blue (2,000,000) was used to determine the void volume (V_o).

Polyacrylamide gel electrophoresis

For examining the homogeneity of the enzymic preparations, electrophoresis under non-denaturing conditions was performed in 10% (w/v) acrylamide slab gel according to the method of Davis (1964) using a Tris-glycine buffer, pH 8.3. Gels were stained with Coomassie Brilliant Blue R-250 dye. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using slab gel of 10% acrylamide in a Tris-glycine buffer pH 9.2 containing 0.1% (w/v) SDS was carried out to estimate subunits of enzyme under denaturing conditions (Laemmli, 1970). The following standard proteins were used for molecular weight determination: SDS-denatured α-lactalbumin (14,400), soybean trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (45,000) bovine serum albumin (66,700) and phosphorylase b (97,000).

Enzyme characterization

A K_m value of ASPG II was estimated from Lineweaver-Burk plot by relating initial reaction velocities to different L-asparagine concentrations. Estimates of pH and optimal temperature of ASPG II were made by using a pH range from 5.0-9.5 (intervals 0.5) and temperature range from 10-80°C, 10°C intervals. The thermal stability was also investigated by measuring the residual activity of the enzyme after 15 min of incubation at different temperatures ranging from 30-90°C, 10°C intervals prior to substrate addition. The substrate specificity of asparaginase toward different substrate was determined. The effect of metal cations (Ni^{2+}, Co^{2+}, Hg^{2+}, Ba^{2+}, Zn^{2+}, and Mn^{2+}) was performed by incubating the enzyme for 15 min at 37°C with 5 mM of cations prior to substrate addition. The percentage of relative activity was calculated by the activity at any point of the curve over highest activity at the optimum point.

RESULTS

Asparaginase activities in five Egyptian cowpea cultivar seeds and germinating seeds are shown in Table 1. In all cultivars, the specific activity of asparaginase was higher in germinating seeds. The highest specific activity was observed in *V. unguiculata* cv. Fodder. For this reason, it was decided to purify the asparaginase from the Fodder cultivar germinating seeds.

Chromatography of the crude extract of the Fodder germinating seeds on a DEAE-sepharose column resolved the asparaginase into three forms, named ASPG I, ASPG II, and ASPG III eluted with 0.0, 0.1, 0.2 NaCl stepwise (Fig. 1). The specific activities of ASPG I, ASPG II, and ASPG III were 43.5, 511, and 82 units per mg protein which represents 0.42, 4.89, and 0.79 fold purification, respectively (Table 2). So, it was decided to focus on ASPG II that shows the highest asparaginase activity.

The ASPG II from DEAE-sepharose was purified by using gel filtration. Two peaks of protein were observed (Fig. 2). One of them has aparaginase activity. The molecular mass of the native asparaginase was estimated to be 70 kDa by gel filtration. When protein was denaturated and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE); the molecular mass was 35 kDa indicating this enzyme has two similar subunits (Fig. 3).

Table 1. Asparaginase activities in *V. unguiculata* from different cultivars

Cultivars	Specific activity of seeds (units/mg protein)	Specific activity of germinating seeds* (units/mg protein)
Kareem 7	18.5 ± 0.73	74 ± 2.16
Dokki 331	14.3 ± 0.31	49 ± 1.71
Kafer El-Sheikh 1	24.3 ± 0.56	87 ± 1.96
Kaha 1	16.8 ± 0.82	63 ± 2.18
Fodder	32.3 ± 0.86	105 ± 3.40

* Germination of seeds after 7 days
Each value is the mean ± s.e. for the three determinations

Table 2. Purification scheme for *V. unguiculata* cv. Fodder asparaginase

Step	Total activity (units)	Total protein (mg)	S.A. (units/mg protein)	Fold purification	Recovery (%)
Crud extract	857	8.2	104.5	1	100
Dialysis against 50 mM potassium Phosphate buffer pH 8	842	8.1	103.9	0.99	98
DEAE-Sepharose					
ASPG I	183	4.2	43.5	0.42	21.3
ASPG II	394	0.77	511	4.89	46
ASPG III	110	1.33	82	0.79	13
Sucrose conc. of asp II	380	0.76	500	4.78	41
Sephacryl S-200					
ASPG II	310	0.32	969	9.27	36

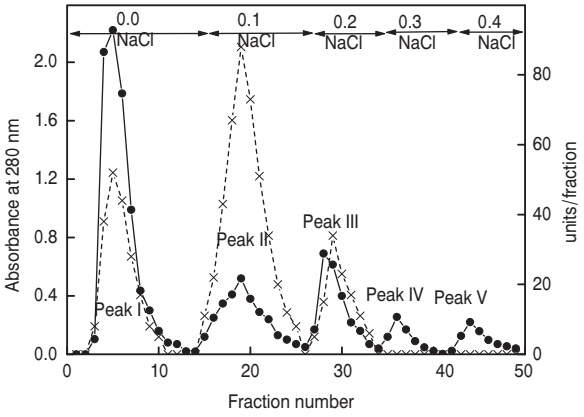

FIG. 1. A typical elution profile for the chromatography of *V. unguiculata* cv. Fodder asparaginase on DEAE-sepharose column (3.5×1.6 cm i.d.) previously equilibrated with 50 mM sodium phosphate buffer, pH 7.5 at a flow rate of 30 ml/hr (.——.), absorbance at 280 nm for protein, (x-----x), asparaginase activity.

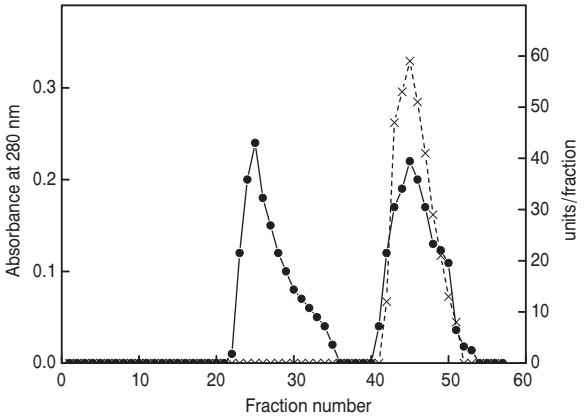

FIG. 2. A typical elution profile for *V. unguiculata* cv. Fodder asparaginase ASPG II DEAE-Sepharose fraction on Sephacryl S-200 column (90×1.6 cm i.d.) previously equilibrated with 50 mM sodium phosphate buffer, pH 7.5 at a flow rate of 20ml/hr (.——.), absorbance at 280 nm for protein, (x-----x), asparaginase activity.

Table 3. Specificity of ASPG II activity toward different substrates and the effect of divalent cations on ASPG II

Substrate	% Relative activity	Divalent cation*	% Relative activity
L-asparagine	100	Ni^{2+}	139
D-asparagine	0	Ba^{2+}	25
L-glutamine	36	Zn^{2+}	36
L-aspartyl hydroxamate	60	Hg^{2+}	20
L-glutamyl hydroxamate	16	Mn^{2+}	42
		Co^{2+}	134

* The enzyme was preincubated for 15 min at 37°C with 5 mM of listed metal cations as final concentration prior to substrate addition. The activity in absence of metal cations was considered as 100% activity

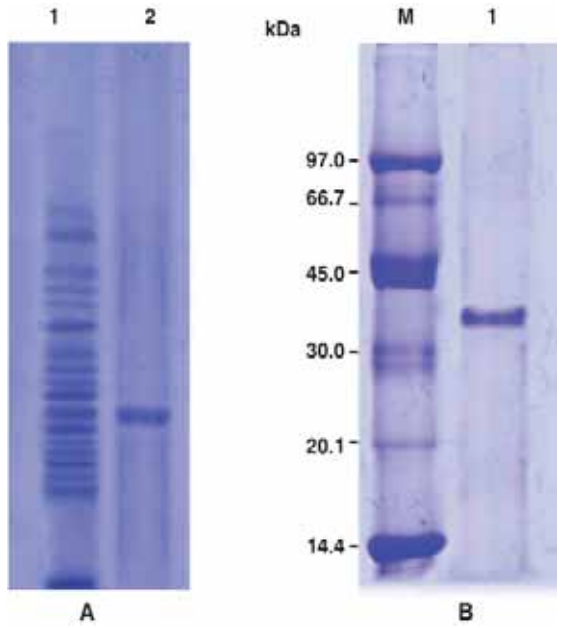

FIG. 3. (A) Polyacrylamide gel electrophoresis for asparaginase from *V. unguiculata* cv. Fodder during purification step. 1: crude extract; 2: Sephacryl S-200 ASPG II. (B) SDS-PAGE for molecular weight determination of asparaginase. M: Standard proteins; 1: Sephacryl S-200 ASPG II.

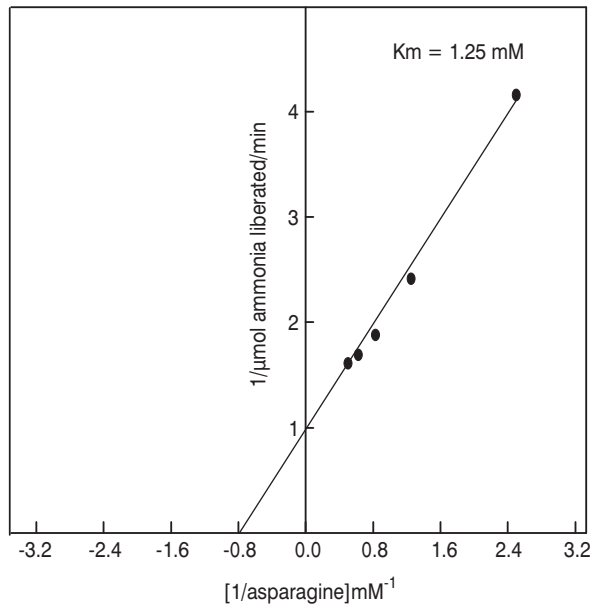

FIG. 4. Lineweaver-Burk plot relating ASPG II, reaction velocities to different L-asparagine concentrations. Each point represents the average of two experiments.

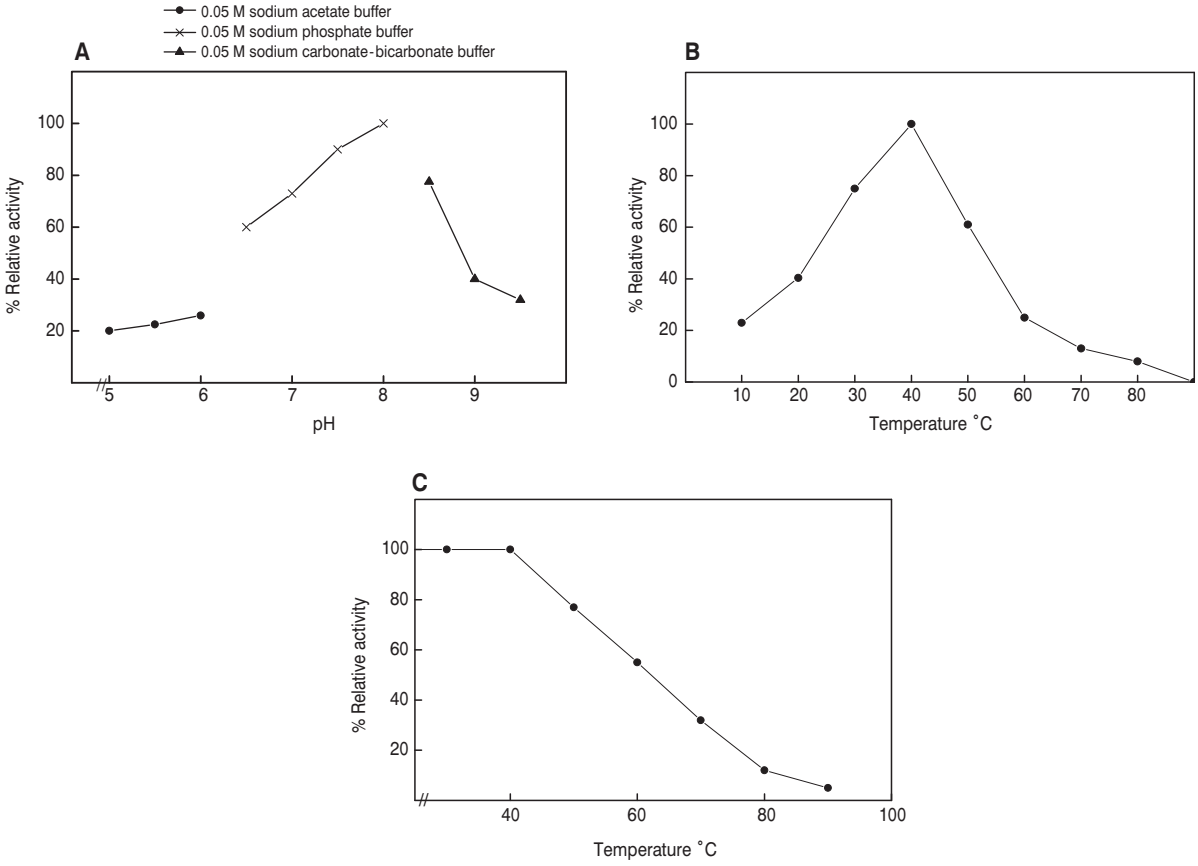

FIG. 5. pH optimum (A), temperature optimum (B), and temperature stability (C) of ASPG II.

K_m, pH dependence and the effects of temperature on ASPGII

The K_m of asparaginase was 1.25 mM, for asparagine as substrate (Fig. 4). The pH optimum of ASPG II was found at pH 8.0 at 50 mM phosphate buffer (Fig. 5A) and the temperature optimum at 40°C (Fig. 5B). ASPG II activity was stable up to 40°C followed by sharp decline. ASPG II lost about 95% of its activity at 90°C (Fig. 5C).

Substrate specificity and the effect of cations on ASPGII

The relative activity of the purified ASPG II hydrolyzing L-aspartyl hydroxamate, L-glutamine and L-glutamyl hydroxamate was found to be 60, 36, and 16%, respectively. The enzyme did not hydrolyze D-asparagine (Table 3). Ni^{2+} and Co^{2+} had activator effects on the activity of ASPG II while Hg^{2+}, Ba^{2+}, Zn^{2+}, and Mn^{2+} had inhibitory effects reducing ASPG II relative activity to 20, 25, 36, and 42%, respectively (Table 3).

DISCUSSION

Asparaginase has been reported in some legume plants such as lupin (Lough *et al.*, 1992), pea (Murray & Kennedy, 1980; Sodek *et al.,* 1980) and soy bean (Streeter, 1977). In this investigation, asparaginase was quantitatively screened in 5 cultivars of *V. unguiculata* (Kareem 7, Dokki 331, Kafer El-Sheikh 1, Kaha 1, and Fodder). In all cultivars, the specific activity of asparaginase was higher in germinating than non-germinating seeds. Borek & Jaskolski (2001) have reported that asparaginase activity in legume seeds increases during cotyledon maturation and reaches a maximum either just before or at the time of maximum protein synthesis. The developmental changes in activity of asparaginase enzyme in any legume embryo and young leaves indicates that early embryo development relies on the seed coat for amino acid supplies. Fait *et al.* (2006) have shown that *Arabidopsis* sp. seeds accumulate a pool of free asparagine at the end of seed maturation, and that asparaginase transcript early during seed germination. This might indicate that the enzyme may metabolize the stored nitrogen, in early stages of germination.

Chromatography of germinating seeds of *V. unguiculata* cv. Fodder crude extract on a DEAE sepharose column resolved asparaginase into three protein fractions. The highest asparaginase activity was detected for ASPG II. Three isoforms of asparaginase were also obtained from *Lupinus abroresus* (Lough *et al.*, 1992). Khushoo *et al.* (2004) separated two isoenzymes of L-asparaginase from *E. coli,* while only one form was detected in other microorganisms like *Vibrio succinogenes* (Distasio *et al.*, 1976), *Enterobacter aerogenes* (Mukherjee *et al.*, 1999) and *Erwinia carotovora* (Kamble *et al.*, 2006).

The Fodder cultivar ASPG II consist of two subunits with 35 kDa molecular mass each. The molecular mass of aspraginase from *Lupinus* species ranged from 70 to 75 kDa with two subunits (Table 4). On the other hand, the *E. coli* L-asparaginase II protein is composed of four identical subunits and has molecular weight of 141 kDa (Khushoo *et al.*, 2004). The enzyme in *Erwinia aroideae* has an apparent molecular mass of approximately 155 kDa and has four subunits of identical molecular mass of approximately 38 kDa (Tiwari & Dua, 1996). One peptide subunit with molecular weight 160 kDa was observed in *Pseudomonas aeruginosa* (El-Bessoumy *et al.*, 2004).

The K_m value for the Fodder cultivar ASPG II was 1.25 mM using asparagine as substrate. This asparaginase has higher affinity to asparagine substrate than aspraginase purified from *Lupinus* species (ranged from 6.6 to 7, Table 4), or *Pisum sativum* testa and cotyledons (3.2 and 3.7 mM, respectively; Sodek *et al.*, 1980). L-asparaginase of various microorganisms had different substrate affinities; K_m values for L-asparaginase from *E. coli* and *Erwinia carotovora* were 2.5 and 7.14 mM, respectively (Willis & Wool-

Table 4. Comparison between asparaginase enzyme from *Vigna unguiculata* and different *Lupinus* species on molecular weight, K_m and pH

Isolated from	Molecular weight (kDa)	No. monomers	K_m (mM)	pH optimum	Reference
Vigna unguiculata	70	2	1.25	8.0	Present study
Lupinus abroresus	75	2	6.60	8.0	Chang & Farnden, 1981
Lupinus angustifolius	—	—	7	8.5	Chang & Farnden, 1981
Lupinus polyphyllus	75	2	—	—	Lea *et al.*, 1984

folk, 1974; Kamble *et al.*, 2006). On the other hand, low K_m values (0.026, 0.047, and 0.147 mM) were obtained for L-asparaginase from *Protus vulgaris*, *Vibrio succinogenes* and *Vibrio aeruginosa*, respectively (Tosa *et al.*, 1972; Distasio *et al.,* 1976; El-Bessoumy *et al.*, 2004).

Vigna unguiculata cv. Fodder ASPG II had pH optimum at 8 in 50 mM phosphate buffer. This value is close to those reported for other asparaginases purified from *Lupinus* species (pH optimum at 8-8.5, Table 4). However, the pH optimum of L-asparaginases purified from various microorganisms ranged from 8.6 to 9.0 (Iiboshi *et al.*, 1999; Balcão *et al.*, 2001; El-Bessoumy *et al.*, 2004; Kamble *et al.*, 2006).

The optimum temperature of the Fodder cultivar ASPG II activity was recorded at 40˚C. This optimum is similar to that reported for *E. coli* and *Pseudomonas aeruginosa* (Qian *et al.*, 1996, El-Bessoumy *et al.*, 2004). On the other hand, the highest reaction rate of L-asparaginase from Chrombacteriaceae and *Proteus vulgaris* was observed at 20˚C and 57˚C, respectively (Roberts *et al.*, 1972; Tosa *et al.,* 1972). The Fodder cultivars ASPG II was stable up to 40˚C and lost 30% of its activity after incubation at 50˚C for 15 min. L-asparaginase from *Pseudomonas aeruginosa* lost 52% of its activity at 50˚C (El-Bessoumy *et al.*, 2004). *Escherichia coli* L-asparaginase lost activity more rapidly than L-asparaginase from *Pseudomonas aeruginosa* at higher temperatures (Qian *et al.*, 1996).

Vigna unguiculata cv. Fodder ASPG II was very specific for L-asparagine and did not hydrolyze D-asparagine. The enzyme showed low activity not only toward L-glutamine but also toward analogue L-glutamine (L-glutamyl hydroxamate). *Lupinus* asparaginase hydrolyzed only L-asparagine and DL-aspartyl hydroxamate but not glutamine (Chang & Farnden, 1981). The animal and microorganism asparaginases catalyze the hydrolysis of both asparagine and glutamine to varying degrees (Borek & Jaskolski, 2001). Several chemical properties make asparagine a convenient vehicle for N transport. It is more soluble than ureides and more stable than glutamine and also a more efficient N carrier than glutamine because it has higher N/C ratio (Canãs *et al*., 2007). Asparagine as a transport molecule, appears to be targeted toward sink tissues where large amounts of nitrogen are needed. Glutamine dependent asparagine synthase activity is the main source for asparagine for nitrogen transport in plants (Azevedo *et al.,* 2006; Canãs *et al.,* 2007).

Vigna unguiculata cv. Fodder ASPG II was activated by Ni^{2+} and Co^{2+} and was inhibited by Mn^{2+}, Zn^{2+}, Ba^{2+}, and Hg^{2+}. It has been reported that metal ions such as Zn^{2+}, Hg^{2+}, and Ni^{2+} potentially inhibit L-asparaginase isolated from *Pseudomonas stutzeri* (Manna *et al.*, 1995) and from *Cylindrocarpon obtusisporum* (Rash *et al.*, 1990).

In conclusion, *V. unguiculata* asparaginase differs in many respects from asparaginases purified from microorganisms. The characteristic of purified asparaginase from Egyptian cowpea may be a potential candidate for chemotherapy. Further study will be carried out to investigate the anti-tumor activity of the isolated asparaginase.

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