Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 127 - 137

Research Article **Bio chemistry**

International Journal of Pharma and Bio Sciences

ISSN 0975-6299

CHARACTERIZATION OF THE A-AMYLASE INHIBITOR FROM THE SEEDS OF *MACROTYLOMA UNIFLORUM* AND *VIGNA UNGUICULATA*

LAXMI H. GUPTA[1], ASHOK CHOUGALE[2], MAHESH KULKARNI[2] AND SUSHMA G. SABHARWAL[1]*

[1]Department of Chemistry, Division of Biochemistry, University of Pune, Pune-411 007
[2]Department of Organic Chemistry, National Chemical Laboratory, Pune-411 008

ABSTRACT

α-amylase inhibitors were purified and characterized from the seeds of *Macrotyloma uniflorum* (MUAI) and *Vigna unguiculata* (VUAI). MUAI was purified using CM-cellulose column while VUAI was purified using Poros HS-50 column followed by gel filtration on Sephadex G-75 column. The molecular weights of MUAI and VUAI as determined by gel filtration were 26.91 kD and 21.89 kD respectively. Both MUAI and VUAI were found to be glycoproteins. The secondary structure of both MUAI and VUAI was determined using circular dichroism studies at different pH and different temperatures. MUAI and VUAI were identified using MALDI-TOF/TOF and LC MS^E analysis respectively.

KEYWORDS: α-amylase inhibitor, Circular dichroism, glycoprotein, MALDI-TOF/TOF, TLC

SUSHMA G. SABHARWAL
Department of Chemistry, Division of Biochemistry,
University of Pune, Pune-411 007

INTRODUCTION

α-Amylases catalyze the hydrolysis of the α-1,4 glycosidic linkage of carbohydrates. α-Amylase inhibitors retard partially or completely the hydrolysis of starch and other oligosaccharides by α-amylases. Inhibition of these enzymes can be used as a target for the regulation of various physiological processes [1]. These amylase inhibitors from plants have been studied extensively as they confer resistance to plants against insect and microbial pests. Also these inhibitors differ in their specificities towards α-amylases from various sources and hence can be used as tools for engineering plants that are resistant to pests. Some α-amylase inhibitors exhibit high affinity for both mammalian and insect α-amylases while others recognize either insect or mammalian α-amylases [2]. α-Amylase inhibitors present in microorganisms, higher plants and animals have applications in crop protection as well as in the treatment of certain disorders like type II diabetes [3]. Both *Macrotyloma uniflorum* commonly known as Horse gram and *Vigna unguiculata* commonly known as Cowpea are legume crops that can be easily cultivated in dry land areas or low rainfall regions [4,5]. Leguminous plants are a rich source of proteins and peptides that are involved in plant defense including proteinaceous amylase inhibitors [6]. α-Amylase inhibitors from several cereals and legumes have been well characterized with respect to their structures and inhibitory potential [7]. The present work describes the isolation and characterization of the α-amylase inhibitor from the seeds of *Macrotyloma uniflorum* and *Vigna unguiculata* respectively.

MATERIALS AND METHODS

The seeds of *Macrotyloma uniflorum* and *Vigna unguiculata* were purchased locally. All chemicals used were of analytical grade.

(i) Isolation and purification of the α-amylase inhibitors from the seeds of Macrotyloma uniflorum and Vigna unguiculata

The seed meals of both seeds were prepared in physiological saline (0.145 M) respectively, followed by ammonium sulphate fractionation (30-80%). The proteins precipitating between this range were collected by centrifugation at 8,000 rpm for 15 min, dissolved in minimum amount of distilled water, dialyzed against distilled water and finally against acetate buffer (pH 5.4, 10 mM). The dialyzed protein was centrifuged at 8,000 rpm for 15 min and the clear supernatant (Fraction A) was used for further purification of the inhibitor. *Macrotyloma uniflorum* α-amylase inhibitor (MUAI) was purified essentially according to the procedure described by Sabharwal and Devanhalli[8]. Fraction A was subjected to ion-exchange chromatography on carboxy methyl cellulose (CMC) column using acetate buffer (pH 5.4, 10 mM) followed by gel filtration on Sephadex G-75 column. *Vigna unguiculata* amylase inhibitor (VUAI) was purified from the seeds by the procedure of Gupta et al [5]. Fraction A was subjected to ion-exchange chromatography on Poros HS-50 column using acetate buffer (pH 5.4, 10 mM) followed by gel filtration on Sephadex G-75 column.

(ii) Molecular weight determination

The molecular weights of the purified α-amylase inhibitors was determined by SDS-PAGE according to the procedure described by Laemmli [9] . Standard molecular weight markers (Bangalore Genei) were run simultaneously.

(iii) α-amylase inhibitory assay

The inhibitory activity of MUAI and VUAI on the human salivary α-amylase was studied essentially according to the procedure of Bernfeld (1955) [10]. 1.5 U of human salivary α-amylase were preincubated with MUAI (0.1 ml, 100 µg) or VUAI (0.1 ml, 70 µg) in buffer (10 mM phosphate buffer, pH 6.9) at 37°C for 60 min in a total reaction volume of 0.5 ml. α-Amylase reaction was initiated by the addition of 1% soluble starch. Controls without the inhibitors were run simultaneously. The reducing sugar was estimated using the dinitro salicylic acid (DNSA) method [11]. The percent inhibitory activity was calculated by the following formula [12],

Inhibitory activity (%) = $(O.D._{control} - O.D._{test} | O.D._{control}) \times 100$

Lineweaver–Burke plots were drawn for the uninhibited and the partially inhibited human salivary amylase. The rate of α-amylase activity was determined at different starch concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml) in a total digest of 1.0 ml containing phosphate buffer (pH 6.9, 10 mM) and the α-amylase.

(iv) Carbohydrate content

The total carbohydrate content of the MUAI and VUAI were quantitatively determined by the method of Dubois et al (1956) [13]. Phenol-sulphuric acid positive carbohydrates were expressed as percentage of glucose as determined from a standard calibration curve using 0.1 mg/ml solution of glucose. An experiment was conducted in triplicates.

(v) Periodic acid Schiff (PAS) staining

The glycoprotein nature of the pure α-amylase inhibitors was determined by PAS staining which was performed essentially according to the procedure described by Rosenberg I.M. (2005) [14] using Schiff's (Fuchsin-sulphite) reagent for staining until dark pink bands were observed against a faint pink background.

(vi)Determination of the glycan moieties of MUAI and VUAI:

Pure α-amylase inhibitor (0.5mg/ml) was hydrazinolyzed with 0.2 ml of anhydrous hydrazine (1% w/v in toluene) for 8 hours at 100°C in polypropylene screw-capped vials according to the procedure described by Fakuda et al (1976) [15]. A column of Dowex 50Wx2 (H^+ form, L= 30 cm, Φ= 2 cm, capacity 50 ml) prepared as described by Boas (1953) [16] was used for the separation of the sugars in the hydrazinolysate. The hydrazinolysate was passed through Dowex column and the neutral sugars were eluted with distilled water (2 ml fractions). Each fraction was assayed for total carbohydrate content by phenol-sulphuric acid test [13]. The fractions showing maximum carbohydrate content were pooled, lyophilized and used for sugar identification by thin layer chromatography (TLC). Sugar moieties were then analysed by spotting the carbohydrate rich fraction on ready to use TLC plates (Merck). The solvent system used for running the chromatogram was isopropanol : water (8:2, v/v). The chromatogram was developed by using $KMnO_4$ (0.5%) in 0.1 M NaOH as the spray reagent, until bright yellow spots were visible against a pink background [17]. Standard sugars viz. D-glucose, D-arabinose, D-galactose, D-ribose, D-mannose, D-xylose and N-acetyl-D-glucosamine were run simultaneously.

(vii) Circular Dichroism (CD) studies

CD measurements were carried out using a JASCO J-815 spectropolarimeter equipped with a Peltier system for temperature control. All the measurements were carried out at 20°C using a cell with a path length of 0.2 cm. The CD spectra were obtained in the far U.V. region i.e. 190 to 240 nm with a bandwidth of 1 nm, a scanning speed of 100 nm/minute, 3 accumulations and a response time of 1 second. The CD spectra were presented in terms of the molar ellipticity (θ) expressed in $deg.cm^2.dmol^{-1}$. The spectra were deconvoluted by using the reference data of model peptides generated by Reed and Reed (1997) [18] available on the spectropolarimeter. The CD spectrum was recorded in phosphate buffer (pH 7.0, 5 mM) unless otherwise specified. Thermal denaturation studies of MUAI (0.1 mg/ml) and VUAI (0.1 mg/ml) were carried out at different temperatures (20, 40, 60 and 80°C). The conformational changes in the secondary structures of MUAI (0.1 mg/ml) and VUAI (0.1 mg/ml) were studied at different pH using 5 mM buffers (Glycine-HCl pH 2.0, Acetate buffer pH 4.0, Phosphate buffer pH 7.0 or Tris-HCl pH 9.0 buffer).

(viii) MALDI-TOF/TOF analysis of MUAI

MUAI was subjected to in-gel tryptic digestion according to the method described by Shevchenko et al (2007) [19]. Tryptic digest was mixed with the matrix (α-cyano hydroxy cinnamic acid) in a 1:1 ratio and 0.5µl was spotted onto a MALDI target plate. The spots were then allowed to dry and loaded on the mass spectrometer. The data was acquired using 4800 MALDI-TOF/TOF mass spectrometer (AB Sciex, Framingham, MA) linked with 4000 series explorer software. Prior to sample analysis the instrument was

calibrated using 6 peptide standards purchased from AB Sciex up to 30 ppm accuracy.Spectrum was recorded using 900 laser shots for MS and 2500 laser shots for MS-MS using Nd-YAG laser. The spectrum was submitted to database search using GPS Explorer Software. Peptide peaks were matched against a Swiss-prot database using MASCOT search engine. The Parameters used for database searching were: Fixed modifications: Carbamidomethyl and Variable Modifications: Oxidation (Methionine), Deamidation (NQ); MS tolerance of 75 ppm and MS/MS tolerance of 0.4 ppm; taxonomy Viridiplantae.

(ix) LC-MS^E analysis of VUAI [20]:

2 µL of digested peptides were analyzed by using nano ACQUITY UPLC online coupled to SYNAPT HDMS system (MSE) (Waters Corporation, Milford, USA) equipped with a nanolockspray ion source with flow rate of 300 nL/ min (external lockmass standard: Glu-fibrinopeptide). Peptide samples were injected online onto a 5 µm Symmetry C18 trapping column (180 µm i.d. × 2 cm length) at a flow rate of 15 µL/ min. Peptides were separated by in-line gradient elution onto BEH 130 C18 1.7 µM × 75 µM × 150 mm nanoACQUITY analytical column, at a flow rate of 300 nL/ min using a linear gradient (5 to 40 % of B for 70 minutes) elution of the peptides was carried out (A - 0.1% formic acid in water and B - 0.1% formic acid in acetonitrile). Acquisition was performed in positive V mode in a mass range of 50-1990 m/z with a scan time of 1 second with alternating low (5 eV) and high (15-40 eV) collision energy.Each raw data file was processed using ProteinLynx Global Server V2.3 software (Waters) to generate charge state reduced and deisotoped precursor mass lists as well as associated product ion mass lists for subsequent protein identification and quantification. Each processed file was then searched against the protein database obtained from www. uniprot. org using the IDENTITYE database search algorithm within PLGS 2.3.29.

RESULTS

Bulk of the MUAI was eluted as a major peak at 0.2 M NaCl concentration on CM-cellulose column while VUAI was eluted as a major peak at 1.0 M NaCl concentration on Poros HS-50 column. After gel filtration on Sephadex G-75 column 86.0 and 68.17 fold purification was achieved for MUAI and VUAI respectively (Table 1, 2)

.

Table 1
Summary of purification for MUAI

Purification step	Volume (ml)	Protein (mg/ml)	Activity (units/ml/min)	Specific activity	Fold purification
Saline extract	500	90.0	2.3	0.02	1
Fraction A	100	35.0	1.8	0.051	2.55
CM Cellulose chromatography	20	1.24	1.25	1.008	50.4
Gel filtration	5	0.5	0.86	1.72	86.0

Table 2
Summary of purification for VUAI

Purification step	Volume (ml)	Protein (mg/ml)	Activity (units/ml/min)	Specific activity	Fold Purification
Saline extract	500	115.0	2.15	0.018	1
Fraction A	90	38.0	1.87	0.049	2.73
Poros HS-50 chromatography	25	1.9	1.62	0.85	47.5
Gel filtration	5	0.86	1.05	1.22	68.17

Molecular weights of MUAI and VUAI were found to be about 26.91 kD and 21.89 kD respectively by gel filtration (Fig 1).

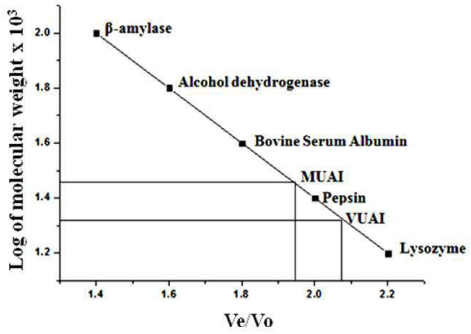


Figure 1
Molecular weight determination by gel filtration on Sephadex G-75 column

SDS PAGE profile of MUAI showed the presence of a single band while the SDS PAGE profile of VUAI showed the presence of 2 bands (Fig 2).

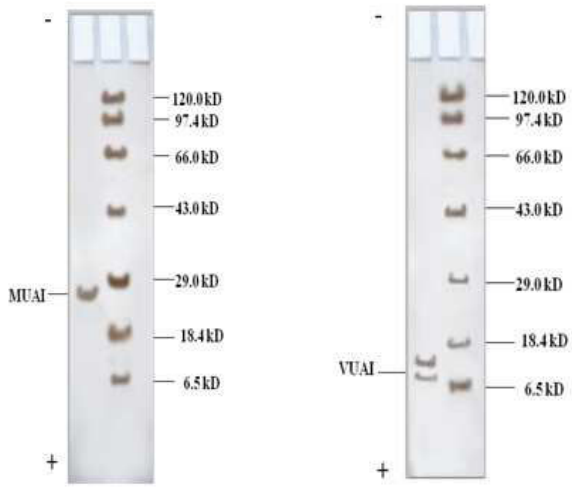


Figure 2
SDS profile of MUAI and VUAI

Both MUAI and VUAI were found to inhibit human salivary amylase in a non-competitive manner with Ki values of 0.66 µM and 3.3 µM respectively (Fig 3).

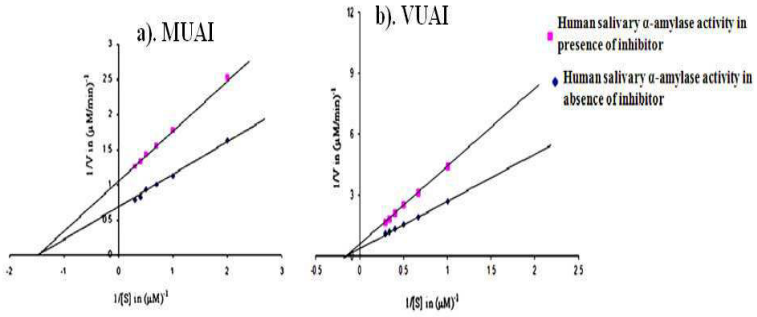


Figure 3
Lineweaver-Burke plots for MUAI and VUAI

The phenol sulphuric acid test revealed that both MUAI and VUAI were glycoproteins with a carbohydrate content of 9.2% and 5.8% respectively. The glycoprotein nature of these inhibitors was further confirmed by their PAS staining gel profile (Fig 4).

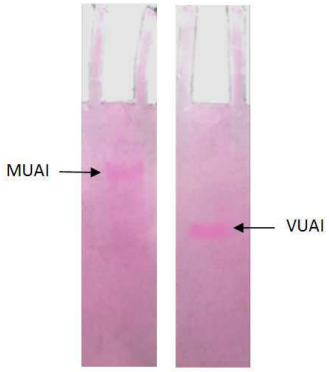
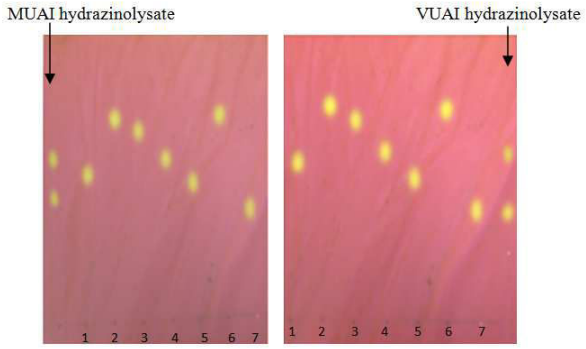


Figure 4
PAS profile of MUAI and VUAI

TLC of MUAI hydrazinolysate showed that the glycan moieties were galactose and mannose while the TLC of VUAI hydrazinolysate showed that the glycan moieties were N-acetyl glucosamine and mannose (Fig 5)



1. Glucose; 2. Ribose; 3. Xylose; 4. Mannose; 5. Galactose; 6. Arabinose; 7. N-acetyl glucosamine

Figure 5
TLC profile for MUAI and VUAI hydrazinolysate

The native conformation of MUAI at 40°C showed 60.3 % β-Sheet, 39.7 % random coils while that of VUAI showed 46.6% α-helix and 53.4 % β-turn conformation (Fig 6, Table 3).

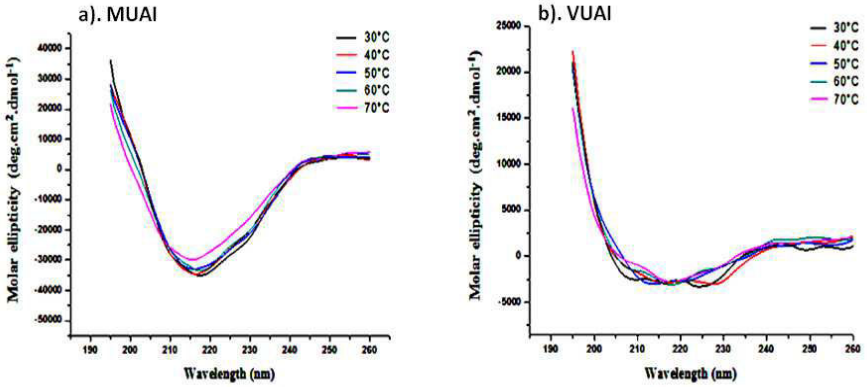


Figure 6
CD spectra at different temperatures

Table 3
Secondary conformation of MUAI and VUAI at different temperatures

	Temperature	α-Helix	β-Sheet	β-Turn	Random coil
MUAI	**30°C**	0.0 %	60.5 %	0.0 %	39.5 %
VUAI		40.1 %	0.0 %	51.6 %	8.3 %
MUAI	**40°C**	0.0 %	60.3 %	0.0 %	39.7 %
VUAI		46.6 %	0.0 %	53.4 %	0.0 %
MUAI	**50°C**	0.0 %	60.1 %	0.0 %	39.9 %
VUAI		41.4 %	0.0 %	58.6 %	0.0 %
MUAI	**60°C**	0.0 %	58.9 %	0.0 %	41.1 %
VUAI		35.0 %	0.0 %	53.9 %	11.1 %
MUAI	**70°C**	0.0 %	57.3 %	0.0 %	42.7 %
VUAI		41.3 %	0.0 %	55.6 %	3.2 %

The native conformation of MUAI at pH 7.0 showed the presence of 35.1 % β-sheet, 15% β-turn and 49.8% random coil while that of VUAI showed the presence of 59.7% β-sheet and 40.3% random coil (Fig 7, Table 4).

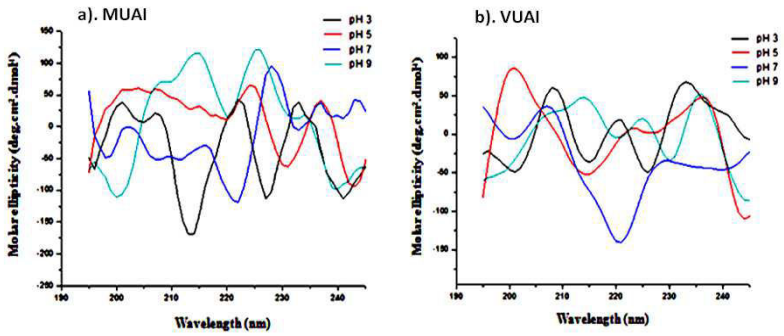


Figure 7
CD spectra at different pH

Table 4
Secondary conformation of MUAI and VUAI at different pH

	pH	α-Helix	β-Sheet	β-Turn	Random coil
MUAI	**3**	0.0 %	56.4 %	0.0 %	43.6 %
VUAI		0.0 %	0.0 %	33.5 %	66.5 %
MUAI	**5**	0.0 %	0.0 %	100.0 %	0.0 %
VUAI		0.0 %	27.9 %	36.8 %	35.3 %
MUAI	**7**	0.0 %	35.1 %	15.0 %	49.8 %
VUAI		0.0 %	59.7 %	0.0 %	40.3 %
MUAI	**9**	0.0 %	0.0 %	39.3 %	60.7 %
VUAI		0.0 %	0.0 %	0.0 %	100.0 %

The MALDI-TOF/TOF analysis (Figure 8) of MUAI and the LC MS^E analysis (Figure 9) of VUAI identified them as defensin like proteins.

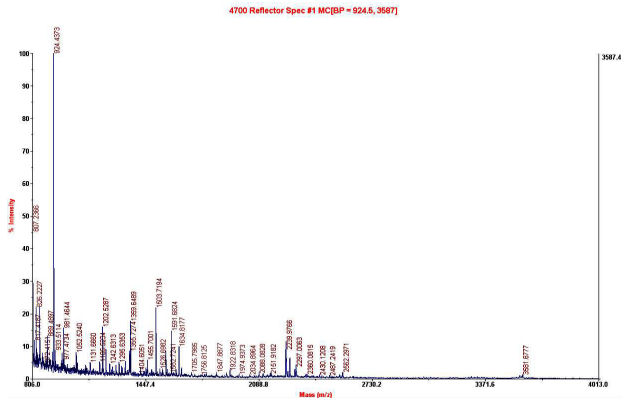


Figure 8
MALDI TOF/TOF spectra of trypsin digested MUAI

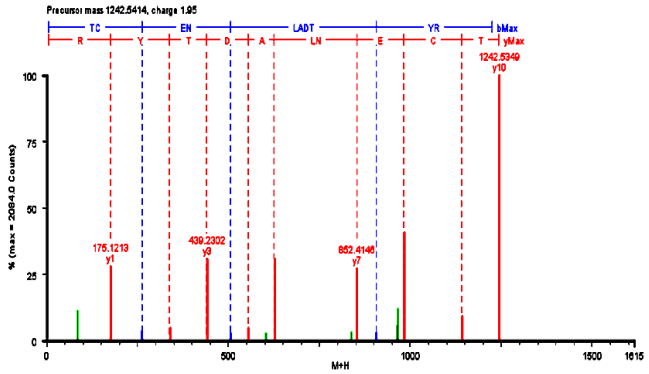


Figure 9
Representative LC MS^E spectra of trypsin digested VUAI

DISCUSSION

The physicochemical characterization and some insights into the secondary structure of the α-amylase inhibitors are useful in understanding the mechanism of inhibition of various α-amylases by these inhibitors and to identify the specificity of an inhibitor for various α- amylases [21].SDS PAGE of MUAI showed the presence of a single band indicating the absence of any interchain disulphide linkages. The SDS PAGE of VUAI showed the presence of 2 bands suggesting the presence of a heterodimer (Fig 2). The

K_i values obtained from the Lineweaver-Burke plots (Fig 3) suggests that MUAI is relatively more potent against human salivary amylase as compared to VUAI under the assay conditions. Characterization of the carbohydrate moieties of glycoproteins is essential since glycosylation is an important posttranslational modification that has a significant impact on a protein's functions [22]. MUAI was found to be a glycoprotein with carbohydrate content of 9.2% which is comparable to that of the α-amylase inhibitors from other legumes such as *Phaseolus vulgaris* [23] whereas the carbohydrate content of VUAI i.e. 5.8% seems to be relatively low**.** β-Sheet proteins have a negative band around 220 nm and a positive band around 200 nm [24]. Increase in β-turns indicates increased protein stability [25]. The change in the intensity observed at 220 nm with increase in temperature is not very significant in case of MUAI, suggesting that it is fairly stable in the temperature range of 30-60°C and thermal denaturation begins above this temperature (Fig 6). At pH 5.0, MUAI showed 100 % β-turn conformation, which may explain its stability in the acidic pH (Table 4). The change in the intensity of the slope at 230 nm is attributed to protein denaturation or unfolding [26]. In case of VUAI, the change in the absorbance at 230 nm at higher temperature indicates unfolding of VUAI (Fig 6). Random coil conformation indicates the absence of an ordered secondary structure in proteins that is required to show their optimum activity [27]. At pH 9.0, the presence of 100% random coiled conformation explains the decrease in activity of VUAI at highly alkaline pH (Table 4).

The MALDI-TOF/TOF analysis of MUAI revealed 80.0% protein score with 'Seed lectin subunit I OS=*Dolichos biflorus* PE=1 SV=2' with a total ion C.I. score of 99.99% (Table 5). The LC MS^E analysis of VUAI revealed 53.19% sequence coverage and a PLGS score of 1167.922 with 'Defensin Fragment OS *Vigna unguiculata* PE 2 SV 1' (Table 6).

Table 5
MALDI-TOF/TOF analysis of MUAI

Entry	Name of protein	Protein score	Total ion C.I. %
LEC1_DOLBI	Seed lectin subunit I OS=*Dolichos biflorus* PE=1 SV=2	80.0 %	99.99

Table 6
LC MS^E analysis of VUAI

	Name of protein	Sequence Coverage	PLGS score	Peptide match with database
B6VEV8	Defensin Fragment OS *Vigna unguiculata* PE 2 SV 1	53.1915	1167.922	(-)KTCENLADTYR(G)
				(K)TCENLADTYR(G)
				(R)CWCTR(N)
				(K)EHLLSGRCR(D)
				(-)KTCENL(A)
				(-)KTCENLA(D)
				(-)KTCENLAD(T)
				(L)ADTYR(G)
				(A)DTYR(G)
				(D)TYR(G)
				(R)CWCTR(N)

CONCLUSION

Both MUAI and VUAI are glycoproteins. MUAI has a high affinity for human salivary amylase and is more thermostable with respect to VUAI.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Srikanth Rapole and Ms. Snigdha Dhali, National Centre for Cell Science (NCCS), Pune, for the help rendered during MALDI-TOF/TOF studies. We also wish to thank Dr. H.N. Gopi, Indian Institute of Science Education and Research (IISER), Pune, for helping with the CD studies.

REFERENCES

1. Doleckova-Maresova L, Pavlik M, Horn M, and Michael Mares, De Novo design of α-amylase inhibitor: a small linear mimetic of macromolecular proteinaceous ligands, Chem Biol, 12: 1349-1357, (2005).
2. Franco OL, Rigden DJ, Melo FR, Carlos Bloch Jr, Silva CP and Grossi de Sa MF, Activity of wheat α-amylase inhibitors towards bruchid α-amylases and structural explanation of observed specificities, Eur J Biochem, 267: 2166-2173, (2000).
3. Sokočević A, Han S and Engels JW, Biophysical characterization of α-amylase inhibitor Parvulustat (Z-2685) and comparison with Tendamistat (HOE-467), Biochim Biophys Acta, 1814 (10):1383-93, (2011).
4. Gupta LH, Badole S, Bodhankar SL and Sabharwal SG, Antidiabetic potential of α-amylase inhibitor from the seeds of *Macrotyloma uniforum* in streptozotocinnicotinamide-induced diabetic mice, *Pharma Bio*, 49: 182-88, (2011).
5. Gupta LH, Raju D, Mehta UJ and Sabharwal SG, A study on the use of α-amylase inhibitor from *Vigna unguiculata* seeds as an organic nitrogen source in plant tissue culture, Int J Pharm Bio Sci, 4(1): (B) 95-105, (2013).
6. Khan N, In vitro Effects of proteinaceous alpha amylase inhibitors on red flour Beetle, *Tribolium castaneum,* Sci Res Reporter 1(2): 10 -104, (2011).
7. Rekha MR, Sasikiran K and Padmaja G, Inhibitor potential of protease and α-amylase inhibitors of sweet potato and taro on the digestive enzymes of root crop storage pests, J stored prod res, 40: 461-470, (2004).
8. Sabharwal SG and Devanhalli AN, Proteinaceous alpha amylase inhibitor from *Macrotyloma uniflorum* seeds: *In vitro* and *in vivo* studies with *Spodoptera litura,* J Ent Res*,* 36 (1): 9-13, (2012).
9. Laemmli UK, Cleavage of structural proteins during the assembly of the head of Bacteriophage T4, Nature, 227: 680-685, (1970).
10. Bernfeld P, Amylases, alpha and beta. *Meth Enzymol*, 1, 149–158, (1955).
11. Sadasivam S and Manickam A, Estimation of reducing sugar by dinitrosalicylic acid method, In: Biochemical Methods, second edition. New Delhi, India: New Age International, 6–7, (2005).
12. Hao X, Li J, Shi Q, Zhang J, He X and Ma H, Characterization of a novel legumin alpha amylase inhibitor from Chickpea (*Cicer arietinum* L.) seeds, Biosci Biotechnol Biochem, 73 (5): 1200-1202, (2009).
13. Dubois M, Gilles KA, Hamilton JK, Reber PA and Smith F, Calorimetric method for determination of sugars and related substances, *Anal Chem.* 28: 350-356, (1956).
14. Rosenberg IM, Protein analysis and purification: Benchtop techniques, Birkhäuser Boston, 2nd edition, 185-186, (2005).
15. Fukuda M, Kondo T and Toshiaki Osawa, Studies on the hydrazinolysis of glycoproteins core structures of oligosaccharides obtained from porcine thyroglobulin and Pineapple stem bromelain, J Biochem, 80:1223-1232, (1976).
16. Boas NF, Method for the determination of hexosamines in tissues, J Biochem, 553-563, (1953).
17. Bhushan R and Kaur S, TLC separation of some common sugars on silica gel plates impregnated with transition metal ions,

Biomed Chromatogr, 11 (1): 59-60, (1997).
18. Reed J, and Reed TA, A set of constructed type spectra for the practical estimation of peptide secondary structure from circular dichroism, Anal Biochem, 254: 36-40, (1997).
19. Shevchenko A, Tomas H, Havlis J, Olsen JV and Mann M, In-gel digestion for mass spectrometric characterization of proteins and proteomes, Nat Protoc, 1(6): 2856 - 2860, (2006).
20. Bhonsle HS, Korwar AM, Kote SS, Golegaonkar SB, Chougale AD, Shaik ML, Dhande NL, Giri AP and Shelgikar KM, Low plasma albumin levels are associated with increased plasma protein glycation and HbA1c in diabetes, J Proteome Res, 11:1391−1396, (2012).
21. Payan F, Structural basis for the inhibition of mammalian and insect a-amylases by plant protein inhibitors, Biochim Biophys Acta, 1696: 171-180, (2004).
22. Lee BS, Krishnanchettiar S, Lateef SS, Lateef NS and Gupta S, Characterization of oligo-saccharide moieties of intact glycoproteins by microwave-assisted partial acid hydrolysis and mass spectrometry, Rapid Commun Mass Spectrom, 19:2629-2635, (2005).
23. Yoshikawa H, Kotaru M, Tanaka C, Kawabata M and Ikeuchi T, Characterization of kintoki bean (*Phaseolus vulgaris*) alpha-amylase inhibitor: inhibitory activities against human salivary and porcine pancreatic alpha-amylases and activity changes by proteolytic digestion, J Home Economics of Japan, 50 (3): 243-23, (1999).
24. Corrêa DH and Ramos CH, The use of circular dichroism spectroscopy to study protein folding, form and function, African J Biochem Res, 3 (5):164-173, (2009).
25. Fu H, Grimsley G, Scholtz JM and Pace CN, Increasing protein stability: Importance of ΔC_p and the denatured state, Protein Sci, 19(5): 1044-1052, (2010).
26. Gopal R, Park JS, Seo CH and Park Y, Applications of circular dichroism for structural analysis of gelatin and antimicrobial peptides, Int J Mol Sci, 13: 3229-3244, (2012).
27. Uversky VN, Natively unfolded proteins: A point where biology waits for physics, Protein Sci, 11(4): 739–756, (2002).