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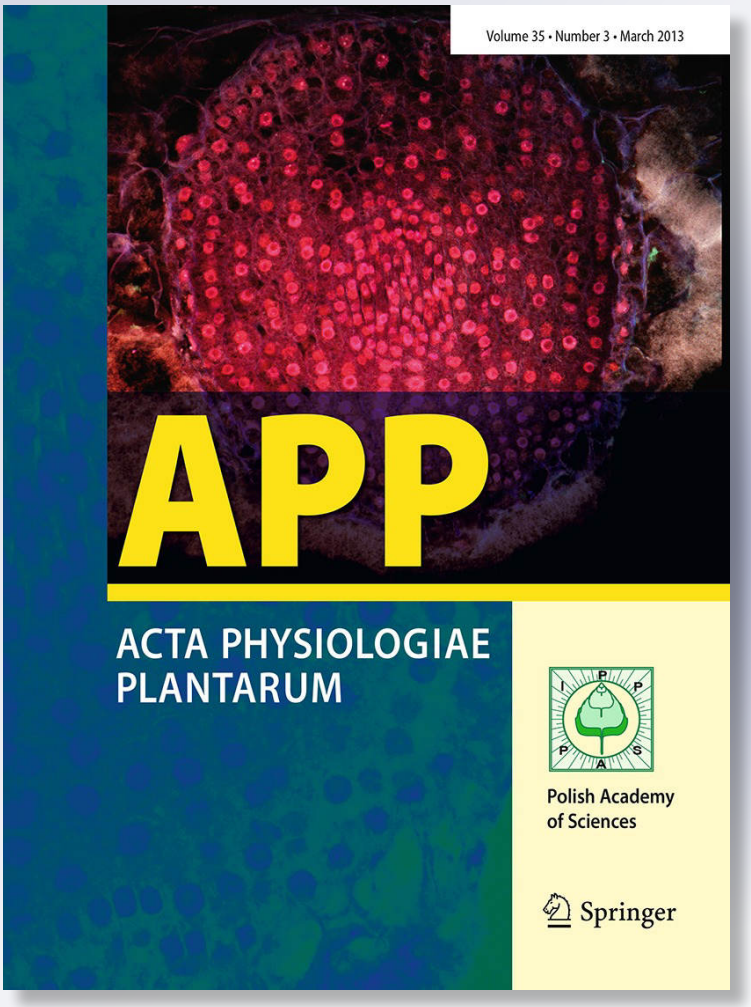

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ORIGINAL PAPER

Novel isoforms of proteinaceous α-amylase inhibitor (α-AI) from seed extract of *Albizia lebbeck*

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Abstract Proteinaceous inhibitors of digestive α-amylase occur naturally in leguminous seeds and find applications in agriculture and clinical studies. We have detected and isolated eight novel α-amylase inhibitor isoforms in the seed extract of *Albizia lebbeck*. They are designated as AL-αAI-1 to AL-αAI-8. These isoforms specifically inhibit human salivary α-amylase and porcine pancreatic α-amylase. The occurrence and profile of α-amylase inhibitor isoforms were revealed by 7 % native-PAGE containing 0.1 % starch. The apparent molecular weights of native bands of AL-αAIs were 97.4, 68.6, 61.0, 57.2, 56.0, 54.7, 51.1, and 47.7 kDa, respectively. Partial purification of potent α-amylase inhibitor was achieved using ammonium sulfate fractionation and gel filtration chromatography on G-100 Sephadex column followed by preparative gel electrophoresis. SDS-PAGE analysis of partially purified AL-αAI showed two polypeptide bands of ~35.8 and ~32.6 kDa. All these isoforms showed effective resistance to in vitro proteolysis by pepsin, trypsin, and chymotrypsin. These inhibitors are stable over a wide range of pH and temperature and have optimum activity at pH 7 and at 37 °C. The finding and information obtained in the present investigation about novel isoforms of α-amylase inhibitors from *A. lebbeck* could be important and may find applications in clinical studies to modulate starch digestion and glycemic index.

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Keywords Proteinaceous α-amylase inhibitor · *Albizia lebbeck* · Human salivary α-amylase · Porcine pancreatic α-amylase

Abbreviations

α-AI	α-Amylase inhibitor
AL-αAI	*Albizia lebbeck* α-amylase inhibitor
BPB	Bromophenol blue
BSA	Bovine serum albumin
DNSA	3,5-Dinitrosalicylic acid
HSA	Human salivary α-amylase
PAGE	Polyacrylamide gel electrophoresis
PPA	Porcine pancreatic α-amylase
PVP	Polyvinylpyrrolidone

Introduction

Proteinaceous α-amylase inhibitors (α-AIs) have been detected in seed extracts of several leguminous plants (Xiaoyan et al. 2009; Krishna et al. 2007; Payan 2004; Yamada et al. 2001; Franco et al. 2000; Giri and Kachole 1998). They are anti-nutritional factors which prevent dietary starches from being absorbed in the digestive tract by inhibiting digestive enzyme α-amylase (Octavio and Rigden 2002). They are grouped into six different classes, namely, lectin-like, knottin-like, cereal-type, Kunitz-like, γ-purothionin-like, and thaumatin-like (Richardson 1991; Franco et al. 2002).

These inhibitors are regulators of endogenous enzymes and active component in plant-defense (Payan 2004; Svensson et al. 2004; Valencia-Jimenez et al. 2000; Franco et al. 2002). α-AIs show great potential as tools to engineer

resistance of crop plants against pests (Svensson et al. 2004; Franco et al. 2002; Octavio and Rigden 2002; Schroeder et al. 1995). They are employed for the study of α-amylase isoenzyme patterns in serum for diagnostic purposes (Huang and Teita 1982; O'Donnell et al. 1977). α-AIs are also known as starch blockers since they play vital role in management post-prandial hyperglycemia in type 2 diabetes complications (Obiro et al. 2009; Celleno et al. 2007; Fowler 2007; Chokshi 2006; Tormo et al. 2004; Leiner et al. 1984).

Several functions of these inhibitors in human and plant physiology tend to find new resources (Svensson et al. 2004; Franco et al. 2002). The first requirement of this study is initial efforts of screening for novel α-amylase inhibitors. With the objective of identifying novel α-AI, we screened several plants and *Albizia lebbeck* seed extract that are good sources.

Albizia lebbeck is a traditional medicinal plant for human. *Albizia lebbeck* belongs to the family Fabaceae, and inhabits entire Indian Subcontinent and other parts of the world. It is used to treat cataract, asthma, ophthalmopathy, leprosy, diarrhea, and poisoning; it has anti-allergic, anti-inflammatory, anticonvulsant, anti-fertility, anti-microbial, anti-arthritic, and anti-oxidative activities (Anthonamma et al. 2010; Saha and Ahmed 2009; Resmi et al. 2006; Gupta et al. 2005; Pratibha et al. 2004; Chintawar et al. 2002; Baruah et al. 2000).

In the present study, we have identified and characterized eight isoforms of α-AI by electrophoretic separation followed by in-gel visualization on polyacrylamide gels. Potent α-AI was partially purified and characterized. Quantitative estimations and studies of biochemical properties were performed using enzyme assays.

Materials and methods

Materials

Dry seeds of *Albizia lebbeck* were collected from Dr. Babasaheb Ambedkar Marathwada University (BAMU) Campus, Aurangabad (MS), India. Electrophoresis system was purchased from Broviga, India. Trypsin (bovine pancreas, E.C. 3.4.21.4), Chymotrypsin (bovine pancreas, E.C. 3.4.21.1), and polyvinylpyrrolidone (PVP) were obtained from Sisco Research Laboratories Pvt. Ltd., India. Porcine pancreas α-amylase, Type I-A (2× crystallized suspension in 2.9 M NaCl containing 3 mM $CaCl_2$), and porcine stomach mucosa pepsin (E.C. 3.4.23.1) were purchased from Sigma Aldrich. Molecular weight markers were purchased from Genei. All other chemicals and reagents were of analytical grade.

Extraction of proteinaceous AL-αAIs

Seeds of *Albizia lebbeck* were ground to fine powder with the help of mortar and pestle and mixer grinder. The flour obtained was defatted with hexane (1:10 w/v) for 3–4 times and air-dried. The protein was overnight extracted in the Mili-Q water containing 1 % polyvinylpyrrolidone (PVP). The flour to water ratio was 1:6 (w/v). The suspension was then centrifuged at 12,000*g* for 20 min at 4 °C and the supernatant (crude extract) was collected.

Specificity characterization of AL-αAIs

For initial specificity characterization of α-AI, in-gel activity staining method following 7.0 % native-PAGE (Davis 1964) was used. HSA and PPA were loaded in each lane without and with the crude seed extract. The constant current (20 mA) was supplied to the gel till the tracking dye "bromophenol blue" (BPB) reaches the bottom of the gel. After electrophoresis gel was placed in a 1 % soluble starch solution in 0.1 M phosphate buffer, pH 6.8, for 1 h at 4 °C and after 1 h, it was carefully rinsed with Milli-Q water. α-amylase activity was observed on the polyacrylamide-starch matrix as clear bands on a blue-colored background after staining with a Lugol's reagent (1 g iodine dissolved in 100 ml 1 M potassium iodide) for 10 min. The absence of clear bands in lanes containing inhibitor with enzyme confirms inhibitory efficacy. The gel was washed, to remove the excess iodine solution and then photographed.

Ammonium sulphate fractionation

The clear supernatant obtained from defatting and extraction (about 40 ml), i.e., $(NH_4)_2SO_4$, was saturated in three stages, i.e., 0–30, 30–60, and 60–90 % at 4 °C overnight. The protein precipitate obtained in each stage was dissolved in minimum quantity of 0.1 M sodium-phosphate buffer, pH 6.8 and dialyzed extensively against the same buffer at 4 °C overnight. The protein obtained was stored in small vials and was used for further studies. The amount of protein present in the crude seed extract, each stage of $(NH_4)_2SO_4$ precipitation and HSA were estimated by the Folin phenol reagent (Lowry et al. 1951) using bovine serum albumin (BSA) as standard.

Detection of AL-αAI isoforms

Crude seed extract and $(NH_4)_2SO_4$ fractions were subjected to 7 % native-PAGE and co-polymerized with 0.1 % starch as described by Giri and Kachole (1998) with little modification. About 100 µg of sample was loaded into each wells of the gel. After the electrophoresis, the gel was

equilibrated with 0.1 M phosphate buffer of pH 6.8 for 10 min, immersed in diluted HSA, and incubated for 30 min at 37 °C. After rinsing with Milli-Q water, it was stained with Lugol's reagent. AL-αAI isoforms were seen as dark bands on the gel where the starch was not digested by the enzyme. The same procedure was carried out for detection of PPA inhibitors from crude seed extract and $(NH_4)_2SO_4$ extract. The apparent molecular weight of native activity bands of AL-αAI isoforms was determined by loading molecular weight markers on the gel.

Partial purification of potent AL-αAI

The dialyzed fraction of 60–90 % $(NH_4)_2SO_4$ showing maximum AL-αAI activity was loaded on a Sephadex G-100 column (30 × 1.8 cm, flow rate 1 ml/3 min) equilibrated with 0.1 M Tris–HCl, pH 7.8. Fractions of 1.5 ml each were collected and monitored for protein (A280) and α-amylase inhibitor activity. The active fractions were pooled and subjected to preparative electrophoresis. After electrophoresis, a vertical strip of gel was cut and processed to detect potent AL-αAI activity band by in-gel detection method as described earlier, with the only difference that starch was not incorporated in gel instead strip of gel dipped in buffered starch prior to enzymatic digestion. A horizontal strip of the remaining gel corresponding to potent AL-αAI activity band was excised and kept overnight at −20 °C and the sample was eluted. The preparative electrophoresis was repeated several times to obtain sufficient amounts of AL-αAI activity band for further characterization. Molecular weight of partially purified AL-αAI activity band was analyzed by 8 % reducing SDS-PAGE (Laemmli 1970).

AL-αAI assay

α-amylase inhibitor activity was assayed based on Bernfeld's method (Bernfeld 1955). Increasing concentration of inhibitor extract was mixed with HSA and PPA in different test-tubes and incubated for 10 min at 37 °C. The reaction was started by adding extract-enzyme mixture to test tubes containing 1 % starch in 0.1 M phosphate buffer of pH 6.8. These tubes were incubated for 10 min at 37 °C and reactions were terminated by adding DNSA (1 % 3,5-Dinitrosalicylic acid, 30 % Sodium potassium tartarate, 0.2 M NaOH) reagent to the assay mixture. The assay tubes were kept in a boiling water bath for 5 min, cooled under tap water and the color of maltose liberated was measured at 540 nm. Controls without inhibitor were run simultaneously. One α-amylase activity unit is defined as the amount of enzyme that will liberate 1 m mol of maltose in 1 min under the assay conditions (pH 6.9, 37 °C). Inhibitory activity is expressed as the percentage of inhibited enzyme activity out of the total enzyme activity.

pH stability and temperature stability

Stability of AL-αAI and partially purified AL-αAI against different H^+ ion concentrations was determined by pre-incubating with PPA in different buffer system, i.e., 0.1 M sodium-citrate pH 4.0, 0.1 M sodium-citrate pH 5.0, 0.1 M sodium-citrate pH 6.0, 0.1 M phosphate buffer pH 7, 0.1 M Tris–HCL pH 8, 0.1 M Tris–HCL pH 9, 0.1 M Glycine-NaOH pH 10, 0.1 M Glycine-NaOH pH 11 for 10 min at 37 °C and α-amylase inhibitor assay was performed according to the standard procedure described previously. Temperature stability of AL-αAI and partially purified AL-αAI studied by pre-incubating inhibitor extract with enzymes at different temperatures, i.e., 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 °C for 10 min.

Effect of proteases

In vitro action of pepsin (15 μg, prepared in 0.01 N HCL) on AL-αAIs (100 μg) and partially purified AL-αAIs was carried out at pH 2.0 at 37 °C for 30, 60, 90, 120, and 150 min in different vials and stopped by the addition of 10 μL of 2 M NaOH. Proteolysis of AL-αAI and partially purified AL-αAI by bovine pancreatic trypsin (20 μg, prepared in 0.1 M Tris–HCL buffer, pH 7.8) and bovine pancreatic chymotrypsin (20 μg, Prepared in 0.1 M Tris–HCL buffer, pH 7.8), was carried by incubating inhibitor extract with these proteases separately in the vials at 37 °C for 30, 60, 90, 120, and 150 min. After these periods of time, the exact volume containing 10 μg inhibitor extract was withdrawn from incubated sample in different vials and used for determination of residual α-amylase inhibitory activity against PPA by solution assay and sample containing 40 μg was used for detection by zymographically. Effect of overnight incubation with these proteases was studied by incubating remaining inhibitor extract at 37 °C and residual inhibitory activity was determined by solution assay.

Statistical analysis

All experiments were conducted and analyzed in triplicate. Means and standard deviations were calculated and compared. Analysis was performed using Microsoft Excel.

Results and discussions

In search of novel proteinaceous α-amylases inhibitors, seed extract of *Albizia lebbeck* is investigated as a possible

source. Detection and isolation of eight different isoforms of protease resistant proteinaceous α-amylase inhibitor with specificity towards HSA and PPA are reported. The present study also describes the partial purification and characterization of potent a-amylase inhibitor.

Extraction of proteins

The presence of high amount of protein in the seed extract of *Albizia lebbeck* plant was reported by many researchers (Olorunsanya et al. 2010; Ndemanisho et al. 2006). 50 mg/ml protein was extracted from defatted seed flour using Milli-Q water containing 1 % PVP which is known to precipitate polyphenols. The total protein was estimated using Lowery method (Lowry et al. 1951).

Characterization of AL-αAIs

The preliminary result of inhibition of α-amylase by the crude seed extract revealed that these inhibitors are able to inhibit the HSA and PPA. HSA and PPA showed activity bands in absence of crude extract but fail to produce activity band patterns in the presence of crude extract suggesting inhibition of these enzymes (Fig. 1) on gel by the method of in-gel activity staining following 7 % native-PAGE as described in "Materials and methods".

AL-αAI isoforms

Protein recovered (83.4 %) in the three stages of ($(NH_4)_2SO_4$) precipitation with crude seed extract was analyzed for α-AI by qualitatively using simple and sensitive in-gel detection method described by Giri and Kachole (1998). The fractions 30–60 and 60–90 % showed inhibition to both HSA and PPA on the gel, whereas fraction 0–30 % did not show inhibition of these enzymes (Figs. 2, 3).

The lane containing crude extract, fractions 30–60 and 60–90 % produced eight isoforms of α-amylase inhibitors against both enzymes on starch-polyacrylamide gel (Figs. 2, 3) which is designated as AL-αAI-1, AL-αAI-2, AL-αAI-3, AL-αAI-4, AL-αAI-5, AL-αAI-6, AL-αAI-7, and AL-αAI-8. Among eight isoforms, AL-αAI-1, AL-αAI-2, and AL-αAI-3 showed prominent activity on the gel. Using the gel detection method four isoforms of α-amylase inhibitors from pigeon pea was reported by Giri and Kachole (1998). They have used higher level of incorporated starch (0.5 %) but in this study, we have found that 0.1 % starch concentration incorporated in the gel was sufficient to achieve maximum resolution of amylase inhibitor bands in agreement with findings of Fontanini et al. (2007). Utmost attempts are made to get best quality sharp bands of AL-αAI. But due to the inherent limitation of the technique blurring of bands at some extent on the gel was observed. Similar results were obtained in

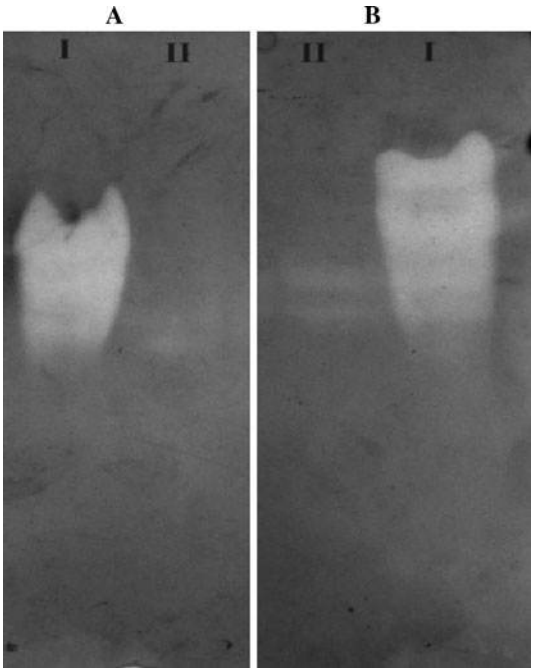

Fig. 1 Zymograms of α-amylase activity (40 µl) in absence (*Lane-I*) and presence (*Lane-II*) of AL-αAI (10 µg) using a 7.0 % native-PAGE **a** salivary α-amylase **b** porcine pancreatic α-amylase

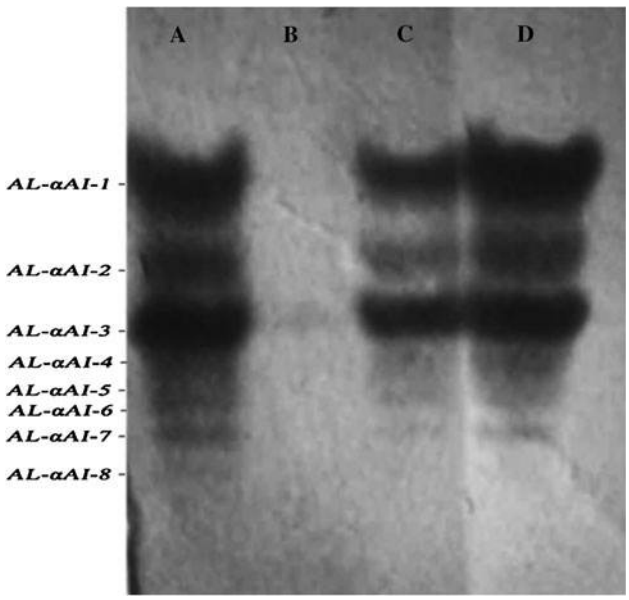

Fig. 2 α-Amylase inhibitors of *Albizia lebbeck* (AL-αAI) against human salivary α-amylase (HSA) on 7 % native-polyacrylamide gel containing 0.1 % starch. **a** Crude seed extract, **b** 0–30 % $(NH_4)_2SO_4$ saturated fraction, **c** 30–60 % $(NH_4)_2SO_4$ saturated fraction, **d** 60–90 % $(NH_4)_2SO_4$ saturated fraction

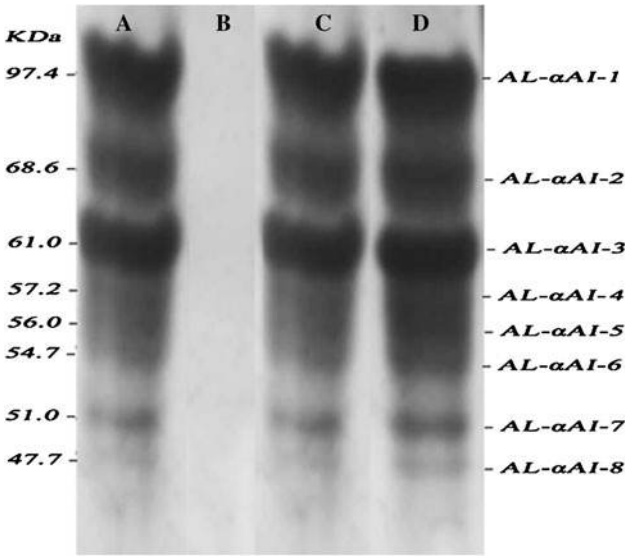

Fig. 3 Starch-polyacrylamide gel of AL-αAIs against porcine pancreatic α-amylase (PPA). Starch-PAGE was performed on 7 % native-PAGE containing 0.1 % soluble starch. **a** Crude seed extracts, **b** 0–30 % $(NH_4)_2SO_4$ saturated fraction, **c** 30–60 % $(NH_4)_2SO_4$ saturated fraction, **d** 60–90 % $(NH_4)_2SO_4$ saturated fraction

the case of α-amylase inhibitors of Pigeon pea (Giri and Kachole 1998).

Partial purification of potent AL-αAI

For the partial purification of AL-αAI, ammonium sulfate ($(NH_4)_2SO_4$) precipitation procedure was used (Kluh et al. 2005; Dayler et al. 2005). They found usefulness of this procedure in isolation and purification of proteinaceous α-amylase inhibitor. Maximum protein was precipitated in 60–90 % $(NH_4)_2SO_4$ precipitation, i.e., 17.2 mg/ml with 14.8 and 9.72 mg/ml in other precipitation stages, i.e., 0–30 % and 30–60 %, respectively. For further purification, the fraction showing maximum AL-αAI activity was loaded on a Sephadex G-100 column equilibrated with 0.1 M phosphate buffer (pH 6.9). Major peak profile of 60–90 % ammonium sulfate-precipitated protein loaded onto a gel filtration column was assessed for AL-αAI activity by solution assay (data not shown). The fractions with higher AL-αAI activity were pulled together and further purified by preparative native–PAGE. The single partially purified band of AL-αAI activity was visualized by in-gel detection method.

Molecular weight analysis

Many workers have documented the presence of high molecular weight α-amylase inhibitors. Molecular weight of purified α-amylase inhibitor of red kidney bean by native-PAGE analysis was 72.6 kDa (Sitthipong 2005). α-amylase inhibitor with molecular weight in the range 45–50 kDa from kidney bean (*P. vulgalis*) was reported by Marshall and Lauda (1975). In the current study, we have found the apparent molecular weight of slow migrating bands, AL-αAI-1 is 97.4 kDa, with remaining is having molecular weights 68.6, 61.0, 57.2, 56.0, 54.7, 51.0, and 47.7 kDa, depending on their migration patterns in the gel with correspond to standard molecular marker (Fig. 3). Partially purified AL-αAI analysis in 8 % SDS-PAGE, in the presence of β-mercaptoethanol, showed two polypeptide bands (Fig. 4) of ~35.8 and ~32.6 kDa on the gel. α-amylase inhibitor having two polypeptide (15.8 and 17.4 kDa) from Palo Fierro seeds (αAI-PF) with high homology to αAI-1 from *Phaseolus vulgaris* was reported earlier (Guzman-Partida et al. 2007).

Quantitative analysis

The $(NH_4)_2SO_4$ fraction (60–90 %) and partially purified AL-αAI were used for the further characterization of these inhibitors. Table 1 shows the percent inhibitory activity of crude AL-αAI against HSA and PPA. 15 µg of the seed extract of *Albizia lebbeck* inhibited HSA by 54.53 % ± 0.6 and PPA by 75.6 % ± 1.1 in solution assay under standard condition. 90.2 % ± 0.75 of inhibition of HSA and 92.4 % ± 1.5 PPA occurred when 25 µg of crude AL-αAI used in solution assay. Furthermore, Table 2 shows the effect of partially purified AL-αAI on HSA and PPA activities. AL-αAI was found highly active against PPA, 1 µg of AL-αAI was required to reach 32.18 ± 1.022 %

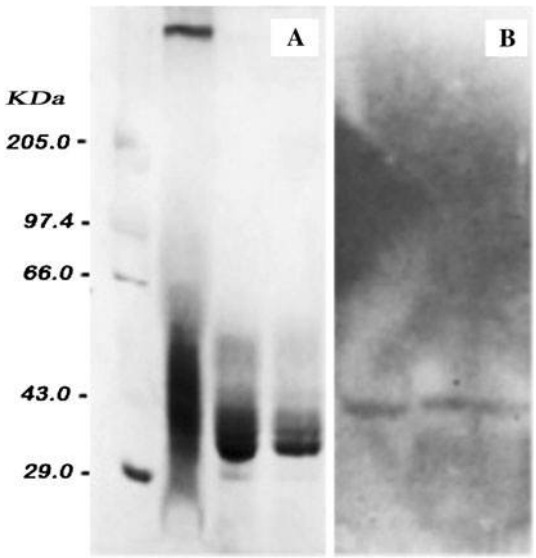

Fig. 4 Electrophoretic analysis of AL-αAI **a** 8 % reducing SDS-PAGE; *Lane-1* Standard Molecular weight marker, *Lane 2* Crude AL-αAI, *Lane 3* Pooled G-100 gel filtration fraction and *Lane 4* Partially purified AL-αAI **b** single activity band of partially purified AL-αAI on 7 % native-polyacrylamide gel containing 0.1 % starch

Table 1 Percent activity of Crude AL-αAI

Sr. no.	Crude seed protein (AL-αAI) in μg	% Inhibition of salivary α-amylase ± SD	% Inhibition of porcine pancreatic α-amylase ± SD
1	5	19.99 ± 0.415	44.88 ± 1.122
2	10	39.71 ± 0.641	52.84 ± 1.209
3	15	54.53 ± 0.671	75.67 ± 1.113
4	20	67.29 ± 0.790	79.88 ± 1.298
5	25	90.22 ± 0.759	92.46 ± 1.556

Increasing concentrations of Crude AL-αAI was incubated with fixed volume (40 μl) of HSA and PPA. Results are presented as mean ± SD, $n = 3$

Table 2 Percent activity of partially purified AL-αAI

Sr. no	Partially purified AL-αAI in μg	% Inhibition of salivary α-amylase ± SD	% Inhibition of porcine pancreatic α-amylase ± SD
1	1	11.29 ± 0.315	32.18 ± 1.022
2	2	21.31 ± 0.431	43.74 ± 0.909
3	3	53.33 ± 0.971	73.27 ± 1.234
4	4	70.09 ± 0.810	73.81 ± 1.098
5	5	88.22 ± 1.459	91.36 ± 1.566

Increasing concentrations of AL-αAI were incubated with fixed volume (40 μl) of HSA and PPA. Results are presented as mean ± SD, $n = 3$

inhibition. On the contrary, at the same concentration AL-αAI was less effective against HSA and reaches 11.29 ± 0.315 % inhibition. Furthermore, 5 μg of AL-αAI was found sufficient for about 90 % inhibition of HSA and PPA, respectively. PPA activity was 100 % inhibited with about 1 μg of αAI-1 from *P. vulgaris*; however, these assays were done at pH 5.5 (Ishimoto and Kitamura 1989).

Biochemical properties

The effect of pH (pH 3-11) on the α-amylase inhibitor activity was studied by pre-incubating AL-αAI and partially purified AL-αAI with HSA (Data not shown) and PPA separately in different buffer system (Fig. 5). The activities of AL-αAI and partially purified AL-αAI were found to be stable in wide pH range from 6 to 9 with optimum at pH 7. They were found stable at 37 °C for several hours like α-amylase inhibitors from pigeon pea reported by Giri and Kachole (1998). Their stability was studied by pre-incubating at different temperatures ranging from 25 °C to 70 °C (Fig. 6). The optimum temperature was at 35 °C. The activities of AL-αAI and partially purified AL-αAI gradually declined at temperatures beyond 60 °C. Minimal inhibition was found at 70 °C indicating denaturation of inhibitors. This finding is in agreement with

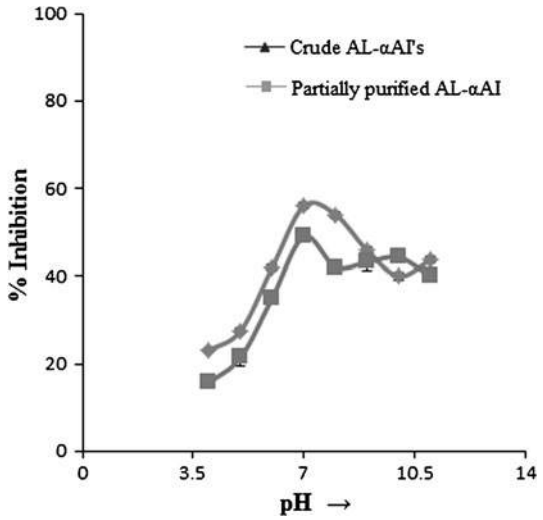

Fig. 5 pH Stability of AL-αAI and partially purified AL-αAI: 10 μg of inhibitor was pre-incubated with HSA and PPA in different buffer systems at 37 °C for 10 min and assayed as described in "Materials and methods". Results are presented as mean ± SD, $n = 3$

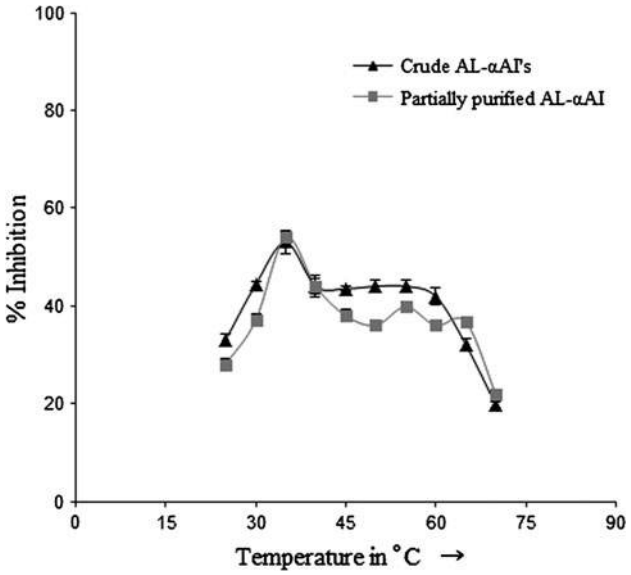

Fig. 6 Thermal stability of AL-αAI and partially purified AL-αAI. 10 μg of inhibitor was pre-incubated at different temperature for 10 min and assayed as described in "Materials and methods". Results are presented as mean ± SD, $n = 3$

that found by LeBerre-Anton et al. (1997) but opposite to the study of Kim et al. (2005) who found that α-AI of pine bark extract was stable at 90–100 °C.

In vitro stability against proteases

The action of proteolytic enzymes on AL-αAI and partially purified AL-αAI activities are shown in Fig. 7. After proteolysis, in-gel detection and solution assay were performed

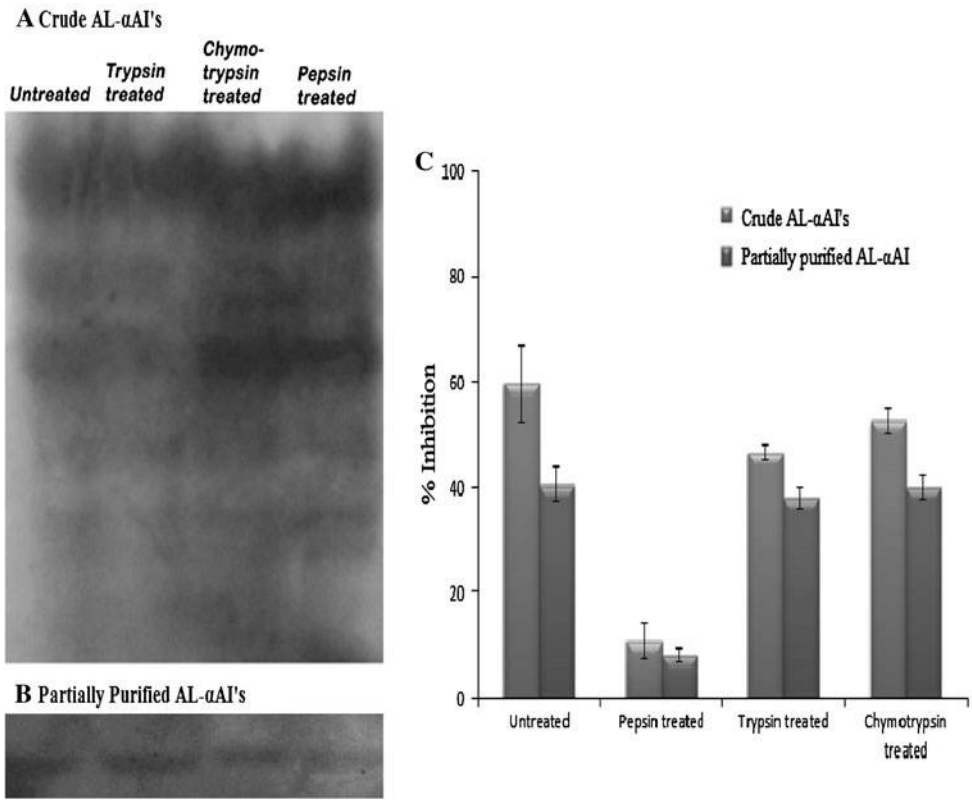

Fig. 7 **a** Crude AL-αAI on 7 % native-PAGE containing 0.1 % starch after incubating for 150 min with inhibitor extracts at 37 °C with porcine stomach pepsin (15 μg), Bovine trypsin (20 μg) and Bovine chymotrypsin (20 μg). **b** Partially purified AL-αAI on 7 % native-PAGE containing 0.1 % starch after incubating for 150 min with inhibitor extracts at 37 °C with porcine stomach pepsin (15 μg), Bovine trypsin (20 μg) and Bovine chymotrypsin (20 μg). **c** Residual percent α-AI activity. Crude AL-αAI and partially purified AL-αAI were incubated overnight at 37 °C with porcine stomach pepsin (15 μg), Bovine trypsin (20 μg), and Bovine chymotrypsin (20 μg). Results are presented as mean ± SD, $n = 3$

against PPA. These inhibitors found to be resistant to the pepsin digestion when incubated at pH 2 for the 150 min in contrast to finding of Nagaraj and Pattabiraman (1985) that pepsin was found to rapidly inactivate the inhibitor and complete abolition of α-AI activity was observed in 20–30 min from proso (*Panicium miliaceum*) seeds. Further, we found that 17 % inhibitor activities still remain on overnight incubation with pepsin in comparison to overnight incubated without pepsin.

AL-αAI and partially purified AL-αAI were found more stable to proteolysis by neutral proteases trypsin and chymotrypsin for 150 min. Furthermore, 75–80 % activities still remain on overnight incubation with trypsin and chymotrypsin in comparison to overnight incubated inhibitor extract without these proteases. In contrast to finding of Yoshikawa et al. (1999) that chymotrypsin reduces inhibitor activity in vitro rapidly within 120 min and slightly by pepsin, we found strong stability of crude AL-αAI as well as partially purified AL-αAI, in agreement with the finding of Gibbs and Ali (1998), which showed that purified α-AI from white kidney beans (*P. vulgaris*) resistant to chymotrypsin and pepsin. Proteolytic action did not release any active peptide which was reported in the case of pigeon pea (Giri and Kachole 1998).

Many studies targeted α-AIs to design drugs for controlling hyperglycemia in type 2 diabetes complications (Obiro et al. 2009; Celleno et al. 2007; Matsui et al. 2007; Chokshi 2006; Layer et al. 1985). Proteolysis of proteinaceous α-AIs in the digestive tracts by pepsin and other proteases limit their utility (Carlson et al. 1983). Furthermore, low inhibitor content in seed extracts does matter (Leiner et al. 1984). PPA has often been used to simulate the human α-amylase because it is structurally and chemically similar to human α-amylase (Qian et al. 2001; Sopade and Gidley 2009). Significant inhibition to PPA by AL-αAI could be useful in targeting α-amylase to design drugs for controlling hyperglycemia. Further strong in vitro stability to proteolysis and high content in seed showed that proteinaceous AL-αAI may find application in a wide clinical research area to modulate starch digestion and glycemic index. Purification of individual isoforms, detailed characterization with kinetic parameters, and in vivo stability of AL-αAIs in the digestive tract will be future prospective of this study.

Author contribution MSK is responsible for the main concept and final approval of the manuscript. MVP is responsible for data interpretation and manuscript writing. FKS designed, performed, and coordinated the main study. AAS helped in sample collection and initial experimentation. PPG helped in electrophoresis and chromatographical techniques. KDS and BVJ helped in data analysis.

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