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Purification and Characterization of a Novel Thermostable Mycelial Lectin from *Aspergillus terricola*

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Abstract Lectin has been isolated from mycelia of *Aspergillus terricola* by single step purification on porcine stomach mucin-Sepharose 4B affinity column. Lectin could be effectively purified with 75% recovery and 4.47-fold increase in specific activity. Lectin migrated as a single band on SDS-PAGE with an apparent molecular mass of 32.5 kDa. Sugar inhibition assay revealed that the lectin did not strongly interact with most carbohydrates and their derivatives tested while strong binding affinity to D-glucose, D-sucrose, *N*-acetyl-D-galactosamine, asialofetuin, porcine stomach mucin, and bovine submaxillary mucin was indicated. Neuraminidase and protease treatment to erythrocytes enhanced lectin titre. Lectin activity was stable within the pH range of 7.0–10.5. *A. terricola* lectin displayed remarkable thermostability and remained unaffected upon incubation at 70 °C for 2.5 h. Lectin did not require metal ions for its activity. Incubation with denaturants (urea, thiourea, and guanidine–HCl) substantially reduced lectin activity. Carbohydrate analysis revealed that it is a glycoprotein with 9.76% total sugars.

Keywords *Aspergillus terricola* · Lectin · Hemagglutination · Inhibition · Purification

Introduction

Lectins are carbohydrate-binding proteins that specifically recognize diverse sugar structures and mediate a variety of biological processes such as cell–cell and host–pathogen interactions, serum–glycoprotein turnover, and innate immune responses [1]. They encompass different members that are diverse in their sequences, structures, binding site architectures, quaternary structures, carbohydrate affinities, and specificities as well as their larger biological roles and potential applications [2]. Almost all cells carry carbohydrates on their surfaces in the form of glycoproteins, glycolipids, and polysaccharides. These carbohydrates have the ability to encode biological information thus serving as

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molecular markers of cell differentiation, development, disease characterization, pathological states, and taxonomic markers of specific microorganisms [3]. Role of lectins in targeting drugs has been reviewed by Tiwary and Singh [4, 5]. With the evidence of their role in several cellular processes, there has been a simultaneous progress in various areas of lectin biology and chemistry [6]. Immense therapeutic potential of lectins from higher fungi has been discussed by Singh and coworkers [7].

The presence of lectins is ubiquitous in nature. They have been isolated from a variety of organisms including viruses, bacteria, fungi, yeast, algae, protozoa, animal, and plant cells [3]. Some of the lower fungi known to elaborate lectins are A*rthrobotrys oligospora* [8], *Rhizopus stolonifer* [9], *Macrophomina phaseolina* [10], *Fusarium solani* [11], *Aspergillus fumigatus* [12], *Aspergillus oryzae* [13, 14], *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus versicolor*, *Aspergillus rugulosus* [15, 16], *Penicillium marneffei* [17], *Penicillium griseofulvum*, and *Penicillium thomii* [18]. In a preliminary investigation on the occurrence of lectins from aspergilli, 15 species of this genus were screened and nine of them (*Aspergillus sydowii*, *Aspergillus candidus*, *Aspergillus allahabadi*, *Aspergillus terricola*, *Aspergillus ficuum*, *Aspergillus sparsus*, *Aspergillus carneus*, *Aspergillus pulvinus*, and *Aspergillus aculeatus*) were found to possess lectins. Due to high hemagglutination activity of *A. terricola*, it was selected for purification and characterization studies.

Materials and Methods

Maintenance, Growth, and Harvesting of Fungal Culture

Fungal strain of *A. terricola* MTCC 3301 was procured from Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India and maintained on czapek agar slants containing (g l^{-1}) sodium nitrate 2.0, potassium chloride 0.5, magnesium sulfate 0.5, ferrous sulfate 0.01, zinc sulfate 0.01, copper sulfate 0.005, dipotassium hydrogen phosphate 1.0, sucrose 30.0, and agar 30.0. The slants were stored at 4±1 °C until further use and subcultured at fortnight intervals. The culture was grown in Erlenmeyer's flasks (1,000 ml) containing 500 ml medium with composition same as that of maintenance medium except agar and incubated at 30 °C for 7 days under stationary conditions. Mycelium was harvested from broth by filtration, washed thoroughly with distilled water, then with phosphate-buffered saline (PBS; 0.1 M, pH 7.2) and pressed dry.

Lectin Extraction

Fungal extract was prepared as described by Singh et al. [15]. Briefly, the recovered mycelium was homogenized in phosphate-buffered saline (0.1 M, pH 7.2) containing 1 mM benzamidine hydrochloride, at high speed (22,000 rpm) for 3–5 min in an ice bath, using an ultra high speed homogenizer (Ultra-Turrax® T25 basic IKA-Werke) followed by grinding with acidified river sand in pestle and mortar for 30 min on an ice bath. The extract was centrifuged at 3,000×*g* for 20 min at 4 °C (Eppendorf Centrifuge 5804 R, Germany) and supernatant was assayed for lectin activity.

Erythrocyte Suspension and Hemagglutination Assay

Blood (type A, B, AB, and O) from human volunteers was drawn in Alsever's solution (ratio 1:2) and centrifuged (400×*g*, 4 °C, 15 min). Erythrocytes were washed thrice in

phosphate-buffered saline (pH 7.2) and resuspended to a concentration of 2% (*v*/*v*). Enzymatic modification of the surface of erythrocytes using neuraminidase (0.2 IU/ml, Sigma Pvt. Ltd., USA) or protease (2 mg/ml, ICN, USA) was performed as described earlier [18]. Agglutination assay was carried out using enzyme-treated and untreated erythrocyte suspension (2%, *v*/*v*) as described previously [15]. Briefly, 20 μl of twofold serially diluted lectin extract was mixed with an equal volume of erythrocyte suspension in wells of U-bottom microtitre plates (Tarsons Products Pvt. Ltd., India). The plates were incubated for 30 min at room temperature and then stabilized at 4 °C for 1–2 h. Hemagglutination was recorded visually when the erythrocytes of control wells (containing PBS and erythrocyte suspension) had settled completely. Lectin titre was defined as the inverse of highest dilution of lectin capable of visible agglutination as depicted by mat formation. The specific activity of the lectin was calculated as titre per milligram protein (Titre/mg).

Hemagglutination Inhibition Assay

Carbohydrates were tested for their ability to inhibit lectin-mediated hemagglutination by incubating appropriately diluted lectin (twice the lowest concentration capable of visible agglutination) with an equal volume of sugar solution in microtitre plates for 1 h at room temperature, followed by addition of 40 μl of human type O erythrocyte suspension (2%, *v*/*v*) to each well. Plates were further incubated for 30 min at room temperature and then stabilized at 4 °C for 2–3 h. Minimum inhibitory concentration (MIC) of sugars capable of inhibiting the lectin-mediated hemagglutination was determined by serial double dilution of the sugar solution. MIC was defined as the lowest concentration of sugar capable of complete inhibition of hemagglutination.

The carbohydrates tested for inhibition were D-ribose, L-rhamnose, D-raffinose, D-xylose, L-fucose, D-fructose, D-mannitol, D-arabinose, L-arabinose, D-galactose, D-glucose, D-mannose, D-sucrose, D-maltose, D-lactose, chondroitin-6-sulfate, inositol, meso-inositol, D-trehalose dihydrate, D-glucosamine hydrochloride, D-galactosamine hydrochloride, D-glucuronic acid, D-galacturonic acid, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, 2-deoxy-D-glucose, 2-deoxy-D-ribose, thiodigalactoside, bovine submaxillary mucin, porcine stomach mucin, asialofetuin, pullulan, melibiose, starch, dextran, and γ-globulin. Simple sugars were tested at a final concentration of 100 mM while glycoproteins and complex sugars were tested at a concentration of 1 mg/ml.

Affinity Purification of *A. terricola* Lectin

CNBr-activated Sepharose 4B (GE Healthcare, USA) was derivatized with porcine stomach mucin (Sigma Pvt. Ltd., USA) following manufacturer's instructions. All purification steps were carried out at 4±1 °C. The affinity matrix previously equilibrated with phosphate-buffered saline (0.1 M, pH 7.2) was packed into 1×10 cm column (GE Healthcare, USA) and lectin extract was loaded at reduced flow rate. Unabsorbed protein was washed with at least five column volumes of phosphate-buffered saline and fractions (1.5 ml) were collected. Protein washout was monitored by reading absorbance at 280 nm (PharmaSpec UV-1700, Shimadzu, Japan) and each of the fractions was assayed for hemagglutination activity using human type O erythrocyte suspension (2%, *v*/*v*). When absorbance of fractions dropped below 0.02, lectin bound to column was eluted with 0.1 M EDTA at reduced flow rate (0.5 ml/min). Elution of lectin was monitored by examining hemagglutination activity and absorbance at 280 nm. Lectin positive fractions were pooled

and dialysed extensively against PBS using Pleated Snake-Skin Dialysis tubing, MWCO 10 kDa (Pierce Biotech., USA). The active fractions after repeated purification were pooled and used for characterization studies. Titre and protein content [19] of the combined fractions was analyzed.

Characterization of Purified Lectin

Purity and Molecular Mass Determination by SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of the combined fractions was run to assess the purity of the sample as described by Laemmli [20] on a Mini-Protean III electrophoretic system (Bio-Rad Laboratories Inc., USA) at a constant voltage (50 V) and protein bands were visualized by staining with Coomasie brilliant blue R 250. Molecular mass of the purified lectin was determined by comparing its electrophoretic mobility with that of standard molecular weight markers (7.0–201.2 kDa; Bio-Rad Laboratories Inc., USA).

pH Optima and Stability

pH optima for lectin activity was determined by carrying out hemagglutination assay using human type O erythrocyte suspension (2%, *v*/*v*) at pH 5.0–9.0 using universal pH buffers. Stability of lectin was determined within the range of pH 1.5–11.5. Purified sample (50 μl) was incubated with 450 μl buffer of respective pH (0.1 M glycine–HCl buffer, pH 1.5–3.5; 0.1 M sodium acetate-acetic acid buffer, pH 4.0–5.0; 0.1 M phosphate buffer, pH 5.5–6.0; 0.1 M Tris–HCl buffer, pH 6.5–8.5; and 0.1 M glycine–NaOH buffer, pH 9.0–11.5) at 4 °C and lectin titre was determined at 0, 2, 4, and 24 h. Samples were neutralized to pH 7.0 prior to titration. Lectin activity at any given pH was expressed as percentage relative activity compared to control (samples incubated with phosphate-buffered saline, pH 7.2 at 4 °C).

Temperature Optima and Thermal Stability

To determine the optimum temperature for activity of *A. terricola* lectin, agglutination assay was carried at 4, 20, 30, and 37 °C. The thermal stability profile of the lectin was deduced by incubating the purified lectin over a temperature range of 25–70 °C with 5 °C increments, in a water bath. Samples were drawn at 0, 10, 30 min, and subsequently at 30 min interval up to 4 h, chilled in ice bath and titrated using human type O erythrocyte suspension (2%, *v*/*v*). Lectin activity at any given temperature was compared to control samples incubated at 4 °C and expressed as percentage relative activity.

Effect of Denaturants and EDTA

Purified lectin was incubated with an equal volume of urea (1–4 M), thiourea (1–4 M), and guanidine–HCl (1–4 M) in PBS at 4 °C for 24 h. Control samples were incubated with an equal volume of PBS at the same temperature. Agglutination assay was carried out at 0, 2, 4, and 24 h using human type O erythrocyte suspension (2%, *v*/*v*). Activity at any concentration at a given time was expressed as percentage relative activity compared to control.

Requirement of divalent ions on lectin activity was determined by extensive dialysis of the purified lectin against EDTA (10 mM) at 4 °C and then against deionized water [21].

Lectin activity was estimated before and after adding 40 mM Ca^{+2}, Mn^{+2}, Mg^{+2}, and Fe^{+2} ions using human type O erythrocyte suspension (2%, *v*/*v*).

Analysis of Total Sugars

Content of total sugars in the purified lectin was estimated by anthrone reagent method using D-glucose as standard [22].

Statistical Analysis

All the experiments were carried out in triplicates and statistical analysis of data was performed using Student's *t* test. Results were expressed as mean of three independent values. The extraction procedures were repeated thrice so as to confirm the reproducibility of results.

Results and Discussion

Affinity Purification of *A. terricola* Lectin

Lectin constituted a major protein in the mycelial extract of *A. terricola.* When crude extract was applied to mucin-Sepharose 4B column, a large peak corresponding to unabsorbed proteins was observed. No activity was however detected in this peak and elution was carried out with 0.1 M EDTA when baseline absorbance dropped below 0.02. Lectin was found to be eluted in three fractions (Fig. 1). Protein content of the combined

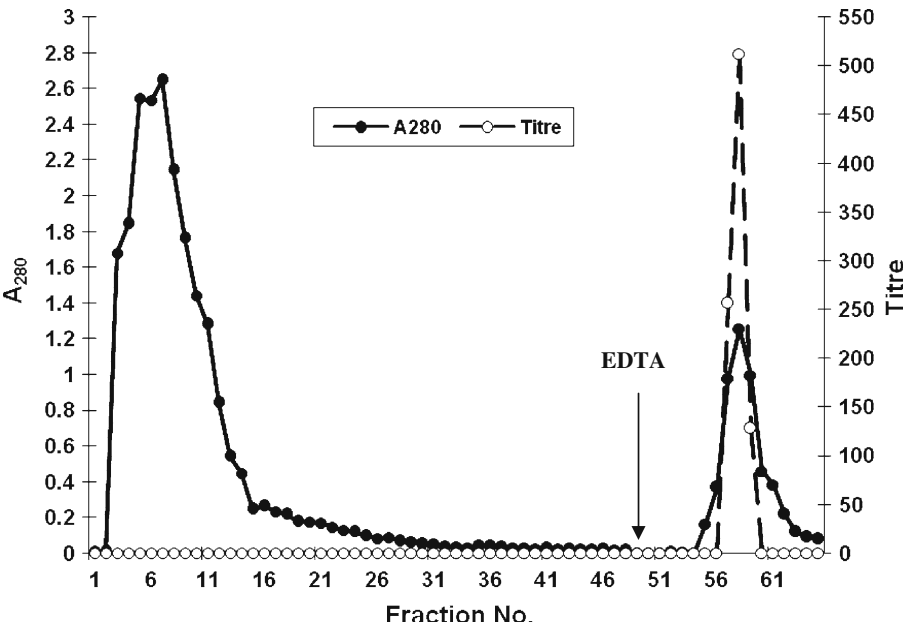

Fig. 1 Elution profile of *A. terricola* mycelial lectin on porcine stomach mucin-Sepharose 4B. Absorbance of fractions was estimated at 280 nm and hemagglutination assay was carried out using human type O erythrocyte suspension (2%, *v*/*v*)

fractions was estimated to be 2.17 mg/ml. About 75% of the total lectin present in the crude extract could be recovered in affinity-purified fractions, reflecting the efficiency of the procedure. A 4.49-fold increase in specific activity was achieved upon purification (Table 1). Single band on SDS-PAGE confirmed the purity of the final product. There are several reports on purification of lectin based on their interactions with specific carbohydrates. *A. oligospora* [8] and *Schizophyllum commune* [23] lectins have also been isolated on porcine stomach mucin-Sepharose 4B column with an overall recovery of 77% and 12.4%, respectively. Lectin from culture filtrate of *M. phaseolina* has been purified by ammonium sulfate precipitation, affinity chromatography on fetuin-Sepharose 4B and ion exchange chromatography on DEAE-A 50 with specific activity of 1,969 titre/mg protein/ml and recovery of 3.25%. Protein content of crude extract was estimated to be 2 mg/ml while that in purified fraction was 0.065 mg/ml [10]. Khan and coworkers [11] reported purification of *F. solani* lectin by three chromatographic steps-two on phenyl sepharose column and one on gel filtration column. Crude extract possessed 32 mg of protein and 2.5 mg protein was recovered upon purification with 3.32-fold purification and 26% recovery yield of hemagglutinating activity. Lectin from *A. fumigatus* has been purified by gel filtration, ion exchange, and hydrophobic interaction chromatographies. Specific activity of 11.1 U/mg has been achieved with 0.96% recovery [12]. Crude extract from ascomycete fungus *Cordyceps militaris* containing 1,140 mg protein when applied to Superdex G 75 column and then on fetuin-agarose affinity column has been reported to yield specific activity of 8.7 titre/mg protein, total hemagglutinating units of 96, and protein recovery of 1% [24]. The present protocol entailed a single chromatographic step based on lectin-carbohydrate interaction and high lectin recovery validates the efficiency of procedures involved.

Hemagglutination and Inhibition Assay

A. terricola lectin was found to be non-specific with regard to its specificity to human erythrocytes. It could agglutinate all human erythrocytes equally with no type preference. Titre 256 was obtained with all the different human blood type suspensions. Neuraminidase and protease treatment of erythrocytes augmented lectin titre by 128 and fourfold, respectively. Neuraminidase treatment cleaves the terminal sialic acid residues and exposes sub-terminal galactosyl moieties thereby reducing the net negative charge on the cells, increasing their ability to be agglutinated [25]. Protease treatment is known to expose the cryptic antigens on the surface of cells which might serve as better ligands [26]. Most of the simple sugars and their derivatives failed to inhibit the activity of *A. terricola* lectin while D-glucose, D-sucrose, and *N*-acetyl-D-galactosamine were inhibitory (Table 2). L-fucose, a common inhibitor of *Aspergillus* lectins [13–15], was not a favorable inhibitor of *A. terricola* lectin (MIC 100 mM). Bovine submaxillary mucin and porcine stomach mucin, containing *O*-linked glycans and asialofetuin containing *O*- as well as *N*-glycosidically linked sugar chains demonstrated a strong binding affinity to the lectin. *N*-glycosidic

Table 1 Summary of purification of *A. terricola* lectin.

Step	Total titre	Total protein (mg)	Specific activity (titre/mg)	Purification fold	Yield (%)
Crude	1,536	58.44±2.39	26.28±1.09	1	100
Affinity purified	1,152	9.76±1.37	118.03±7.57	4.47±0.64	75

Data expressed as mean ± SD; no statistical difference was observed in titre values; agglutination assay was carried out using human type O erythrocyte suspension (2%, *v*/*v*)

Table 2 Minimum inhibitory concentration (MIC) of sugars specific to *A. terricola*.

Sugar	MIC
L-rhamnose	>100 mM
L-fucose	>100 mM
D-arabinose	>100 mM
L-arabinose	>100 mM
D-glucose	>6.25 mM
D-fructose	>100 mM
D-mannitol	>100 mM
D-maltose	>100 mM
D-sucrose	>12.50 μg/ml
Inositol	>100 mM
D-trehalose dihydrate	>100 mM
D-glucosamine hydrochloride	>100 mM
D-galactosamine hydrochloride	>100 mM
N-acetyl-D-glucosamine	>100 mM
N-acetyl-D-galactosamine	>3.12 mM
Asialofetuin	>31.25 μg/ml
Bovine submaxillary mucin	>62.50 μg/ml
Porcine stomach mucin	>3.97 μg/ml

Agglutination studies were carried out using human type O erythrocyte suspension (2%, *v*/*v*)

oligosaccharide chains contain mannose, *N*-acetylglucosamine and galactose [27], none of which were found to be inhibitory for lectin activity, thus indicating that the lectin mainly recognizes sugars present in the *O*-linked chains of asialofetuin. Lectin exhibited several fold higher affinity to porcine stomach mucin (MIC 3.97 μg/ml) than bovine submaxillary mucin (MIC 62.50 μg/ml). Higher titre of *A. terricola* lectin with neuraminidase-treated erythrocytes and its sugar inhibition profile indicate that galactosyl-glycoproteins might mediate the lectin recognition process. Lectin recognizing glycoproteins possessing *N*-linked and *O*-linked glycans has also been reported from mycelial extract of the fungi *F. solani* [11], *Sclerotium rolfsii* [28], and the mushrooms *Chlorophyllum molybdites* [29] and *Psathyrella velutina* [30].

Characterization of Purified *A. terricola* Lectin

Lectin migrated as a single band in SDS-PAGE with an apparent molecular mass of 32.5 kDa (Fig. 2). Molecular mass of *A. fumigatus* lectin has been reported to be 32 kDa [12] and that of *A. oryzae* to be 35 kDa [14], which resemble closely the molecular mass of *A. terricola* lectin. Carbohydrate analysis revealed lectin to be a glycoprotein with 9.76% of total sugars.

pH and Thermal Stability of *A. terricola* Lectin

Hemagglutination activity of *A. terricola* changed upon titration in buffers of different pH with maximum activity attainable at pH 6.5–9.0 and temperature 20–37 °C. Upon incubation with buffers at varied pH for 24 h, no activity was recovered at or below pH 5.0

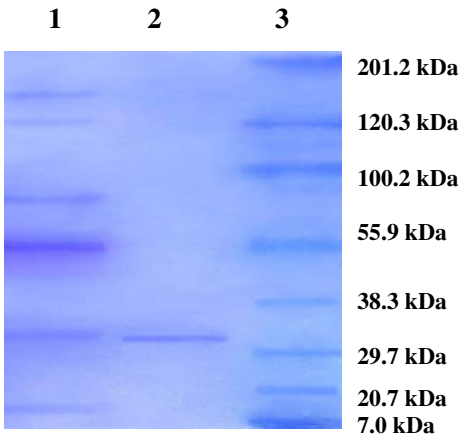

Fig. 2 SDS-PAGE of *A. terricola* lectin using 12.5% gel (running time 4 h at constant 50 V; protein loaded, 40 μg/well). *Lane 1* crude extract, *lane 2* active fractions from porcine stomach mucin-Sepharose 4B column, *lane 3* molecular mass markers from top: myosin (201.2 kDa); β-galactosidase (120.3 kDa); bovine serum albumin (100.2 kDa); ovalbumin (55.9 kDa); carbonic anhydrase (38.3 kDa); soyabean trypsin inhibitor (29.7 kDa); lysozyme (20.7 kDa), and aprotinin (7.0 kDa)

and at pH 11.5. Lectin activity was stable within the pH range of 7.0–10.5 after 24 h while a substantial loss in activity was observed at pH 6.5 after 2 h. pH stability profile of *A. terricola* lectin after 2 h incubation at varied pH is given in Fig. 3. *A. terricola* lectin demonstrated remarkable thermal stability with no affect on activity after 2.5 h incubation at 70 °C. Activity began to decline on further incubation at 70 °C where 6.25% activity was retained after 3.5 h with no activity recovered after 4 h at 70 °C. Complete loss in hemagglutination activity was observed within 1 h at 80 °C. Complete activity was recovered at 65 °C after 4 h. Thermostability of *A. terricola* lectin was more pronounced than earlier reports on lectins from ascomycete fungi [24, 31]. Lectin from *R. stolonifer* has been reported to be stable at 70 °C after 10 min heat treatment [9]. *F. solani* lectin has been accounted to exhibit high stability between pH 2–12 and temperature upto 70 °C for 15 min [11]. Activity of *S. commune* lectin has been reported to be stable at pH 3–10 for 18 h and 55 °C for 30 min [23].

Effect of Denaturants and EDTA on *A. terricola* Lectin Activity

Lectin activity was not affected in presence of 1–2 M urea, with 50% and 75% loss of activity after 24 h incubation with 3 and 4 M urea, respectively. Lectin retained 12.5% and 3.12% activity after 24 h in 3 and 4 M thiourea, respectively. Four molars guanidine–HCl resulted in 93.75% loss after 24 h (Fig. 4). Protein denaturation by these agents indicates globular nature of lectin stabilized mainly by hydrophobic interactions [32].

Metal ion requirement of *A. terricola* lectin was estimated by dialysis of lectin against 10 mM EDTA. Activity remained unaltered after metal ion chelation suggesting that lectin does not require metal ions for binding. Certain lectins require metal ions for their activity while others do not. Our results corroborate with the findings of *R. stolonifer* lectin where EDTA treatment had no affect on its activity [9]. Lectin activity of *F. solani* has been reported to be partially Ca^{+2} dependent as evidenced by considerable reduction in activity after Ca^{+2} depletion by EDTA [10].

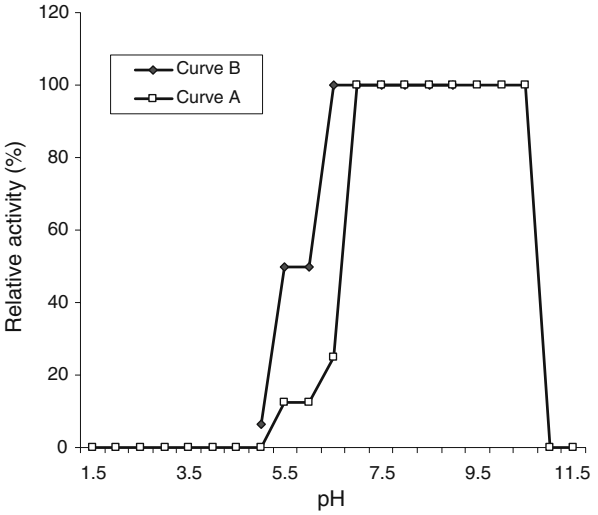

Fig. 3 Influence of pH on lectin activity of *A. terricola*. *Curve A* pH stability profile after 2 h incubation at 4 °C; *curve B* optimum pH; hemagglutination assay was carried out using human type O erythrocyte suspension (2%, *v*/*v*)

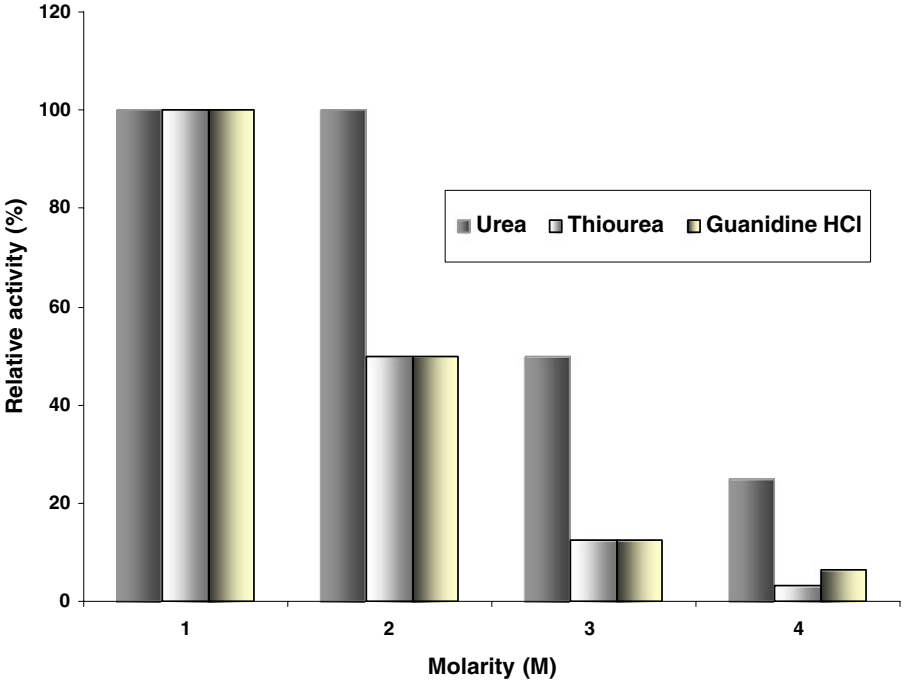

Fig. 4 Effect of denaturants on activity of *A. terricola* lectin after 24 h incubation at 4 °C. Hemagglutination assay was carried out using human type O erythrocyte suspension (2%, *v*/*v*)

Conclusions

This is the first report on lectin from *A. terricola*. The lectin displayed high specific hemagglutination activity. *A. terricola* lectin belongs to the category of panagglutinins or non-specific lectins with respect to its specificity to human erythrocytes. Only fewer sugars could inhibit activity of *A. terricola* lectin unlike lectins from other aspergilli. Sugar inhibition studies suggest that galactosyl type glycoproteins probably serve as binding sites for the lectin. It displays remarkable thermostability and could withstand elevated temperatures over a prolonged period. It did not require any metal ions for binding. The current study adds onto the scanty information available on characteristics of *Aspergillus* lectins.

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