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Isolation, Purification, and Characterization of Two Thermostable Endo-1,4-β-D-glucanase Forms from *Opuntia vulgaris*

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Abstract Four endoglucanase temperature isoforms (T_{30}, T_{50}, T_{70}, and T_{90}) were identified and purified from the cladodes of the xerophytic plant *Opuntia vulgaris*. These isoforms exhibited optimum catalytic activity at 30 °C, 50 °C, 70 °C, and 90 °C and yielded an apparent molecular mass of 150, 20, 74, and 45 kDa, respectively, on gel filtration chromatography. These isoforms were purified 24-, 25-, 29-, and 27-fold with a yield of 15%, 12%, 17%, and 19% and having a specific activity of 120, 125, 144, and 136 U/mg, respectively. The thermostable T_{70} and T_{90} isoforms exhibited optimum activity at pH 4.5 and 7 and also yielded a molecular weight of 66 and 36 kDa, respectively, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The T_{70} had a K_m of 43 mM and a V_{max} of 12.5 μmol min^{-1} μg^{-1} of protein, and the T_{90} isoform had a K_m of 40 mM, with an apparent V_{max} of 10 μmol min^{-1} μg^{-1} of protein. Western blot, immunodiffusion, and in vitro inhibition assays established the reactivity of the T_{90} isoform with polyclonal anti-T_{90} antibody raised in rabbit. Cross-reactivity of this antibody with the T_{70} endoglucanase isoform was also noted.

Keywords Cellulases · Endoglucanase · Thermophilic · Metal ion · *Opuntia vulgaris*

Introduction

Cellulase, also called as endoglucanase, is a multi-subunit enzyme containing a catalytic core, cellulose binding domain, and a flexible, heavily glycosylated linker region [1]. Available cellulosic feedstocks from agriculture and other sources are about 180 million tons per year [2]. Increasing demand for renewable energy sources has sparked growing

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interest in enzymes capable of degrading cellulose to sugars that can then be used for the production of ethanol [3]. The realization that the world's oil supply was finite led to the quest for alternative fuels in 1975, and for economical ways to produce ethanol, from abundantly available, biodegradable, and renewable raw materials [1]. The application of cellulase technology certainly has been drawing more attention than any other aspect. Cellulase is used in the biofuels, although this process is relatively experimental at present. Many have tried to develop a broad scope application for the production of fuels, chemicals, and food. Some of the chemicals are from lignocellulose, hemicellulose, and wood sugar chemicals [4]. Cellulase is used for commercial food processing in coffee, and it also performs hydrolysis of cellulose during drying of beans. Furthermore, cellulases are widely used in textile industry and in laundry detergents. They have also been used in the pulp and paper industry for various purposes, and they are even used for pharmaceutical applications [5]. Cellulase is used as a treatment for phytobezoars, a form of cellulose bezoar found in the human stomach. Cellulases have also been used for the improvement of the soil quality and reduce dependence on mineral fertilizers. Major impediments to the commercial use of cellulases were low activity and the high cost of enzyme production [6]. This necessitated the search for cellulolytic organisms producing cellulase with novel properties as also strategies for low-cost enzyme production.

The identification of the eukaryotic thermostable cellulase reported here is a unique discovery that will satisfy the desirability of industrial needs. The main advantage of a thermostable enzyme over that of a thermolabile enzyme is that the rate of reaction increased as the temperature of the process is increased [7]. A 10 °C raise in temperature approximately doubled the reaction rate and decreased the amount of enzyme required for the catalytic conversion process [8]. The existence of temperature-stable enzymes in *Opuntia vulgaris* plant has been identified from our group earlier [9]. The discovery of thermostable cellulose isoforms as reported here in the eukaryote xerophytic *O. vulgaris* plant species led to their isolation, purification, and physiochemical characterization.

Materials and Methods

Collection of Cladode

O. vulgaris cladode tissue was weighed, dethorned, chopped into small pieces, and homogenized in 0.1 M Tris–HCl, pH 7.0, containing 1 mM PMSF using a Waring blender (4 min) to prepare a 20% *w/v* cladode homogenate. The homogenate was centrifuged in a refrigerated Sorvall RC-5C high-speed centrifuge at 10,000×*g* for 20 min to obtain a clear supernatant used as the enzyme source.

Enzyme Assays

The endoglucanase (endo-1-4-β-D-glucanase) activity of the enzyme source was measured by the dinitrosalicylic acid method [10]. The enzyme activity was determined by incubating 1 ml of assay mixture containing 0.5 ml of 1% carboxymethyl cellulose as substrate and 0.2 ml of suitably diluted enzyme in 100 mM Tris–HCl buffer, pH 7, for 30 min at 50 °C. Enzyme and reagent blanks were also simultaneously incubated with the test samples. The intensity of the orange yellow color developed in the sample was determined at 540 nm employing a Shimadzu UV–visible double-beam spectrophotometer. One international unit of the enzyme activity was defined as the amount of enzyme that released 1 μmol of

reducing sugar from carboxymethyl cellulose per minute. The specific activity of the enzyme was established in relation to the total protein content of the enzyme source, determined by the method [11].

Purification of Endoglucanases

Cladode homogenate supernatant was taken for solid ammonium sulfate precipitation to a final concentration of 80%, at room temperature. The protein precipitate was dialysed against 0.1 M Tris–HCl buffer, pH 7. The dialysate was applied to a Dowex-1 anion exchange column (28×0.83 cm) equilibrated with 0.1 M Tris–HCl buffer, pH 7. The adsorbed material was eluted with a linear gradient of sodium chloride in the range of 0–1 M. Protein content and enzyme activity of the collected fractions were determined at 280 and 540 nm, respectively. The active fractions, corresponding to the peak of endoglucanase activity, were combined and loaded onto a Sephadex G-100 (21×0.5 cm) column equilibrated with 0.1 M Tris–HCl buffer, pH 7. Fractions of 2 ml were collected, followed by protein and enzyme activity determination at 280 and 540 nm, respectively.

Enzyme Characterization

Molecular Weight Determination

Native cellulase molecular weight was determined by size exclusion chromatography on Sephadex G-100 column. The molecular weight was determined from a plot of the ratio V_e/V_o of each standard protein vs. log molecular weight of the protein and relating the Ve of the enzyme active fraction to the standard plot. The purity was checked on native polyacrylamide gel electrophoresis (PAGE). The purified enzyme was subsequently used for characterization.

The subunit molecular weight was determined by sodium dodecyl sulfate (SDS)–PAGE employing 10% acrylamide gels using a Mini-PROTEAN 3 electrophoresis cell (Bio-Rad), followed by quantitation of the SDS–PAGE bands. An amount of 10 μg of purified cellulase was applied per lane, and the separated protein bands were stained and visualized using a solution of 0.25% Coomassie Brilliant Blue R-250 in 10% methanol/7% acetic acid followed by destaining in the same solvent mixture [12].

Effect of pH and Temperature on Enzyme Activity

The endoglucanase activity was measured by incubating a fixed volume (200 μl) of the enzyme sample at 37 °C for 30 min in the pH range 3.0–9.0, employing different buffers (0.1 M citrate buffer, pH 3.0–6.0; 0.1 M Tris–HCl buffer, pH 6.0–8.0; and 0.1 M glycine–NaOH, pH 8.0–9.0), followed by enzyme assay as described earlier. Effect of temperature was determined by incubating a fixed volume of enzyme sample in the temperature range of 30–100 °C initially for 10 min at intervals of 10 °C, and by incubating the reaction mixture for 30 min at each temperature before assaying the enzyme activity as described earlier.

Generation of Polyclonal Anti-endoglucanase Rabbit Antibodies

A polyclonal rabbit antibody to the purified T_{90} endoglucanase from *O. vulgaris* was generated using the enzyme protein (0.5 ml, 1 mg/ml protein) having specific activity 2 U/mg as the immunogen mixed with an equal volume of Freund's complete adjuvant. The protein–

adjuvant emulsion (1 ml) was injected on to the dorsal skin of a male rabbit prepared for the immunization process, as several spots. The rabbit was maintained on normal diet for 14 days thereafter. On the 14th day, the animal was given a booster dose (0.5 ml, 1 mg/ml protein) of endoglucanase in equal volume of Freund's incomplete adjuvant. A second booster dose was administered on day 21, and a third booster was given exactly on the 28th day. On day 29, blood was collected from the earlobe of the animal, clotted, and the serum was separated by centrifugation at 700×*g* for 3 min. The serum as a source of the polyclonal rabbit anti-endoglucanase antibody was stored at 4 °C for further use.

Western Blot Analysis

Western blot analysis was carried out as described by Toubin et al. [13]. Equal quantity (50 μg) of sample protein per lane was separated on a SDS–PAGE run at 100 V for 3 h. Electrophoretic transfer of the separated proteins onto a nitrocellulose membrane was then carried out at a constant current of 50 V in 25 mM Tris–HCl, pH 8.6, for 6 h. The nitrocellulose membrane was incubated for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween-20 supplemented with 5% skim milk powder, was processed with a 1:250 dilution of T_{90} endoglucanase antiserum. The membrane was then washed three times (10 min/wash) and incubated for 1 h at room temperature in fresh blocking buffer containing a 1:1,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Bangalore Genei, Bangalore, India) to determine possible species cross-reactivity with the enzyme isoforms. The specific proteins were detected using the TMB/H_2O_2 reaction in the dark.

Immunodiffusion Studies

A 1% agarose in 0.85% NaCl was used to form the gel matrix. Wells were then cut at equidistance in the agarose matrix using a gel cutter. Ten microliters of the rabbit antiserum (300 μg) was applied, followed by the application of 10 μl of purified T_{90} and T_{70} endoglucanase isoforms (>200 μg) into the antigen wells. The immunodiffusion plates were then allowed to develop at 6 °C for 48 h. Immunoprecipitation occurred that stayed visible to the naked eye as a white precipitin line.

Activity Staining of Endoglucanase

The activity staining method was performed as described by Trudel and Asselin [14]. Carboxymethyl cellulose (0.5%) as substrate was boiled and incorporated into the analytical polyacrylamide gel. The isolated endoglucanase protein samples (0.4 U/mg) were run in the substrate incorporated gel. The polyacrylamide gels were stained for 5 min in calcofluor white M2R (0.001% *w*/*v*) and destained in distilled water for at least 30 min. Bands with lytic activity appeared as dark zones following UV illumination.

Congo Red Plate Assay

Endoglucanases were identified employing the congo red plate assay as described [15]. Agar plates were prepared containing 1% carboxymethyl cellulose incorporated into 1.7% *w*/*v* agar in 0.1 M Tris–HCl, pH 7.0. The experimental steps were conducted in sterile condition in an incubator set at 30 °C. The enzyme sample in 5 to 10 μl volume (specific activity, 0.4 U/mg) was applied onto each agar well. Following a 24–48-h incubation period

at 30 °C, the wells were washed off with distilled water and were stained with congo red solution (0.5 mg/ml) for 30–60 min. The gels were soaked in 1 M NaCl until clear yellow zones were detected.

Inhibition Studies

The polyclonal rabbit antiserum was also used in test tube enzyme assays for evaluating the potency of these antibodies to block the endoglucanase activity when assayed in vitro. *O. vulgaris* endoglucanase antiserum (200 μg; used as a control) in each reaction tube was incubated at 4 °C overnight with fixed volumes of purified isoenzymes (T_{70} and T_{90}) in 0.1 M Tris–HCl buffer, pH 7.0. The residual endoglucanase activity was measured in the supernatant after removal by centrifugation (3,000×*g*) of the antibody–antigen complexes and was compared with the control enzyme active samples.

Effect of Metal Ions on Enzyme Activity

Studies investigating the effect of metal ions on the endoglucanase activity were carried out employing divalent cations in their chloride form in 0.1 M Tris–HCl buffer, pH 7, for 30 min and incubating a fixed amount of the enzyme. The metal ions were used at 1, 5, and 10 mM concentration in independent reactions. The endoglucanase activity determined in the absence of the metal ion served as a control.

Substrate Specificity

Pectin, chitin, carboxymethyl cellulose, and cellobiose, each at a concentration of 5 mM, was used as a substrate for the T_{70} and T_{90} isoforms of *O. vulgaris* (0.1 mg/ml) and were assayed at 70 °C and 90 °C as described earlier.

Enzyme Kinetics

Substrate saturation kinetics of the enzyme activity was carried out in reaction tubes containing increasing amount of carboxymethyl cellulose (20–100 μg) and a fixed amount of the endoglucanase isoforms. Assays were carried out at 37 °C as described earlier.

Results and Discussion

Studies employing the xerophytic plant species *O. vulgaris* have indicated the existence of multiple forms of endoglucanase activity in this species. Endoglucanase of *O. vulgaris* was purified by ammonium sulfate precipitation, ion exchange, and Sephadex G-100 gel filtration chromatography as described under "Materials and Methods" Section. In the present study, ion exchange chromatography on Dowex-1 (pH 7.0) (Fig. 1a) and gel filtration chromatography (Fig. 1b–1e) were used successfully to clearly separate endoglucanase isoforms with approximately 25-fold purification.

The purification result of the endoglucanases employing the ion exchange and gel filtration chromatography methods was summarized in Table 1. The purification fold was 28 and 27, respectively, for the T_{70} and T_{90} isoforms of *O. vulgaris*. The yield of T_{70} and T_{90} protein obtained matched well with earlier reports. Strategic improvements in the purification process and the yield of material were possible only if more modern approaches

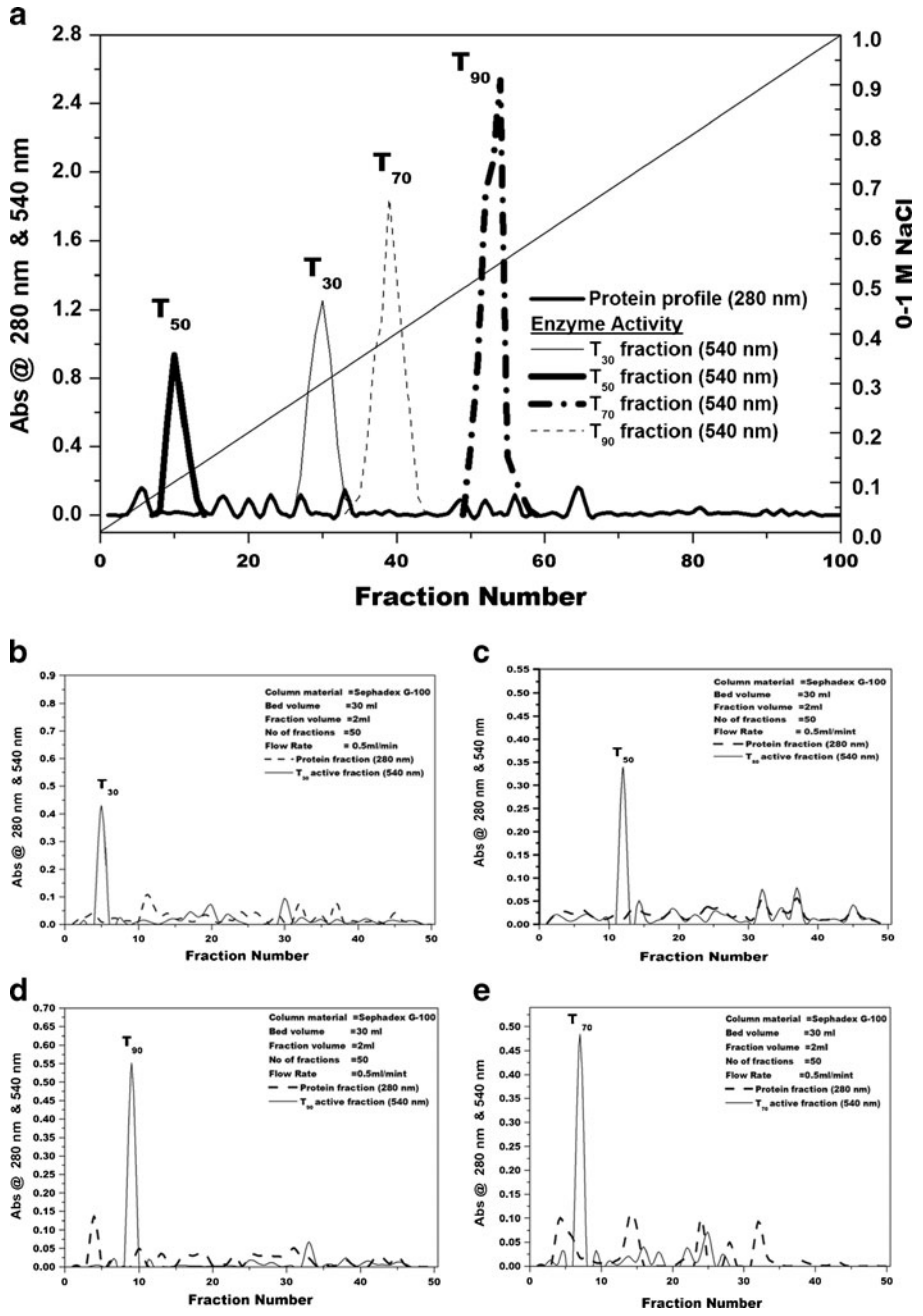


Fig. 1 Elution profile of *Opuntia vulgaris* endo-1,4-β-D-glucanase on Dowex-1 ion exchange (**a**) and Sephadex G-100 chromatography [T_{30} (**b**), T_{50} (**c**), T_{70} (**d**), and T_{90} (**e**) isoform]. Absorbance of fractions was estimated at 280 nm, and endo-1, 4-β-D-glucanase assay was carried out using *O. vulgaris* supernatant (10%, *w*/*v*). The bound enzymes were eluted with a linear gradient of NaCl in ion exchange chromatography

Table 1 Purification of *Opuntia vulgaris* endoglucanase activity

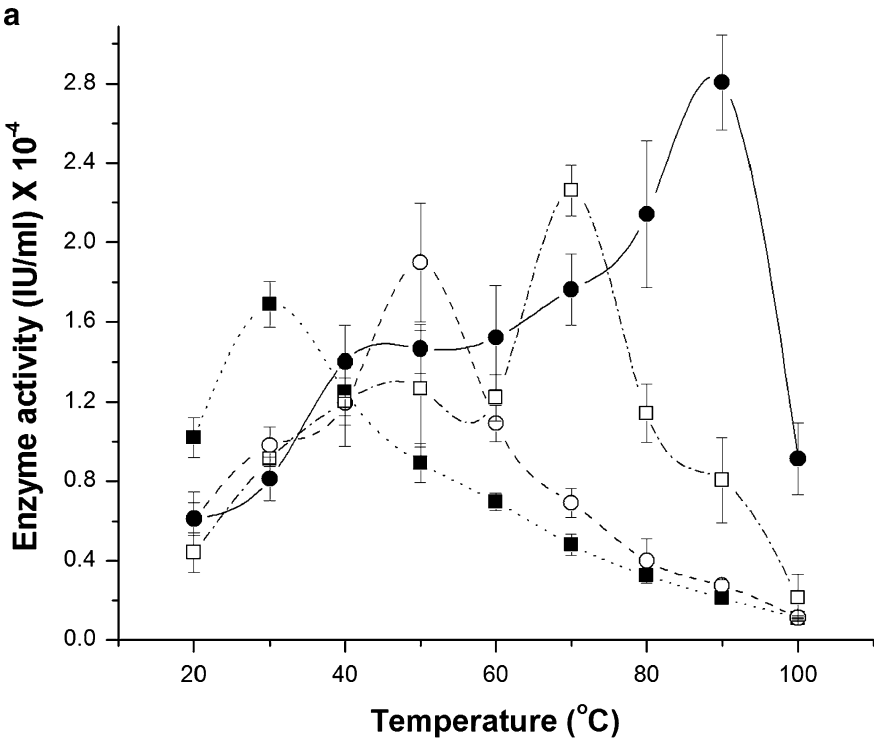
Step	Total protein (mg)	Total activity (units)	Specific activity (U/mg)	Purification fold	Yield (%)
Cladode homogenate	124.0	620.00	5.0	1.0	100
Ammonium sulfate ppt	40.0	300.00	7.5	1.5	48.3
Ion exchange chromatography					
T_{30}	1.8	124.00	68.80	13.70	20.00
T_{50}	1.4	136.00	97.10	19.42	21.90
T_{70}	1.7	162.00	95.20	19.04	26.10
T_{90}	2.0	194.00	97.00	19.40	31.20
Gel filtration chromatography					
T_{30}	0.8	96.00	120.00	24.00	15.40
T_{50}	0.6	75.00	125.00	25.00	12.09
T_{70}	0.75	108.0	144.00	28.80	17.41
T_{90}	0.9	123.0	136.60	27.32	19.80

One international unit of carboxymethyl cellulase activity was defined as the amount of enzyme that released 1 μmol of reducing sugar from carboxymethyl cellulose per minute

had been attempted. The lower recovery of the purified protein is probably due to the sensitivity of certain plant enzymes to extremely low-abundance contaminants like heavy metals present in the chemicals employed for the isolation methods. Most of the purification protocols reported resulted in obtaining homogenous endoglucanase preparations, although the yields were low. This is because multiple steps were needed to get rid of contaminants of endoglucanase activity. Four temperature isoforms (T_{30}, T_{50}, T_{70}, and T_{90}) of endoglucanase corresponding to their temperature optima (30 °C, 50 °C, 70 °C, and 90 °C) have been identified (Fig. 2a). However, among these only two forms, T_{70} and T_{90} were to be considered as thermophilic as per definition. The T_{70} and T_{90} isoforms exhibit optimum activity at pH 4.5 and 7 as shown in Fig. 2b. Since our interest of study was mainly on thermophilic isoforms, characterization of T_{70} and T_{90} was only considered.

The molecular weight of four isoforms T_{30}, T_{50}, T_{70}, and T_{90} of *O. vulgaris* as determined by gel permeation chromatography based on plotting the V_e/V_o ratio of each standard protein vs log molecular weight of the protein (Fig. 3a), was determined to be 150, 20, 74, and 45 kDa. The data suggested the probability of subunit association in the formation of the different temperature isoforms. In SDS–PAGE, the T_{70} isoform was determined to have a molecular weight of 66 kDa for the reduced polypeptide band, while 36 kDa was noted for *O. vulgaris* T_{90} form (Fig. 3b). For example, combination of a 45-kDa T_{90} subunit with 20-kDa T_{50} subunits of the same species may give rise to a 66-kDa T_{70} isoform. Similarly, two 66-kDa T_{70} subunits in association with a 45-kDa T_{90} isoform may give rise to a 150-kDa T_{30} isoform. The differences in the subunit molecular weight of the thermophilic isoforms were indicative of the basic subunit molecular size that made up each isoform since differences in molecular weight determined by SDS–PAGE and gel filtration were indicative as due to the recognition of an extended conformation by the gel filtration technique that generally yielded a higher numeric value for proteins with an extended conformation (non-globular) compared to more compact globular forms under SDS–PAGE conditions.

Molecular weight determination of these two isoforms employing gel filtration technique as well as SDS–PAGE technique yielded similar molecular weights for each of these


a
Enzyme activity (IU/ml) X 10^{-4}
0.0
0.4
0.8
1.2
1.6
2.0
2.4
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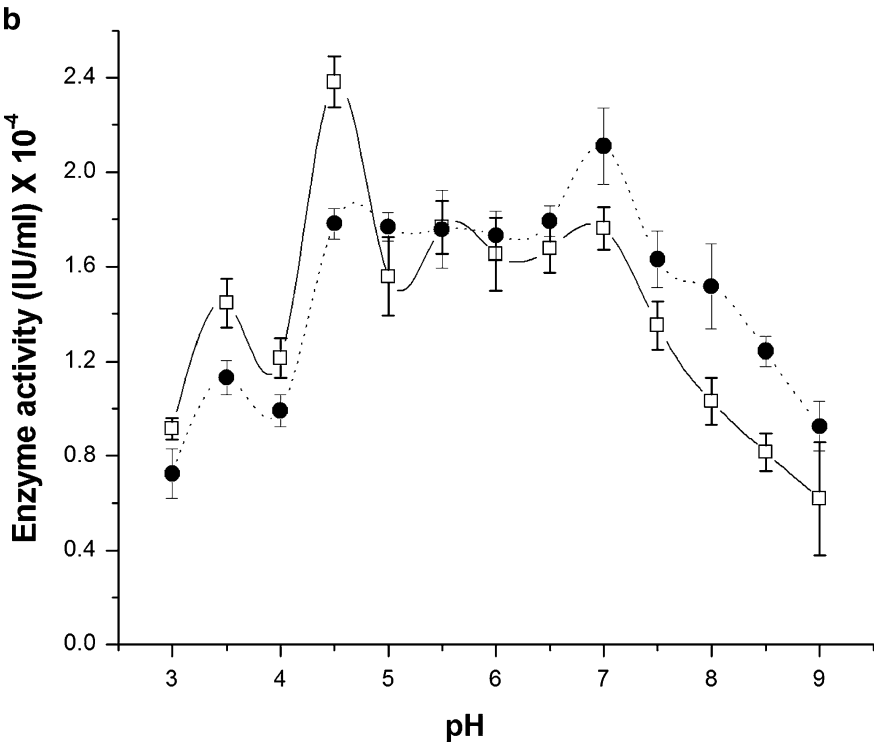
40
60
80
100
Temperature (°C)


b
Enzyme activity (IU/ml) X 10^{-4}
0.0
0.4
0.8
1.2
1.6
2.0
2.4
3
4
5
6
7
8
9
pH

◀ **Fig. 2** **a** Temperature: T_{30} isoform (*black square*), T_{50} isoform (*white circle*), T_{70} isoform (*white square*), T_{90} isoform (*black circle*), and **b** pH profile : T_{70} isoform (*black circle*), T_{90} isoform (*white square*) for purified *Opuntia vulgaris* endo-1, 4-β-D-glucanase isoforms; Enzyme activities were determined as described in the "Materials and Methods" Section. *Values* are the means of those from three independent assays

species. Observations made here are supported by the findings of Luderer et al. [16], who reported on the multiplicity of endoglucanase forms in the fungus *Trichodetma reesei.* Multiplicity of endoglucanase is also reported in *Schizophyllum commune* [17]. The literature survey on purification of cellulases indicated multiple forms of endoglucanases produced by many microorganisms [18]. Eriksson and Pettersson purified and characterized five types of endoglucanases in their studies [19]. The existence of multiple forms of cellulase in crude protein extracts of ripe avocado fruit had been reported by Kanellis [20]. Scrivener and Slaytor [21] observed in their studies that the cellulase of *Panesthia cribrata* consisted of at least six endoglucanase (EC 3.2.1.4) and two β-glucosidase (EC 3.2.1.21) components. Like the mesophilic fungi, the thermophilic fungi produced multiple forms of the cellulase components. Khandke et al., Tong et al., and Ramesh Maheshwari [22–24] described in their studies the two different strains of *Thermoascus aurantiacus* producing one form each of endoglucanase, exoglucanase, and β-glucosidase, but the forms from the two strains have different properties. The multiplicity of individual cellulases might be a result of posttranslational and/or postsecretion modifications of a gene product or might be due to multiple genes. For example, *Talaromyces emersonii* produced multiple endoglucanases, exoglucanases, and β-glucosidases [25]. In *T. emersonii* culture, filtrate protein was resolved by ion exchange chromatography into four endoglucanases which, unlike their variable carbohydrate contents (28% to 51%), had similar molecular masses (68 kDa by gel filtration and 35 kDa by SDS–PAGE), isoelectric points, pH and temperature optima, thermal stabilities, and specific activities [26]. Endoglucanases were also identified employing the congo red plate assay. Appearance of single band in native gel electrophoresis

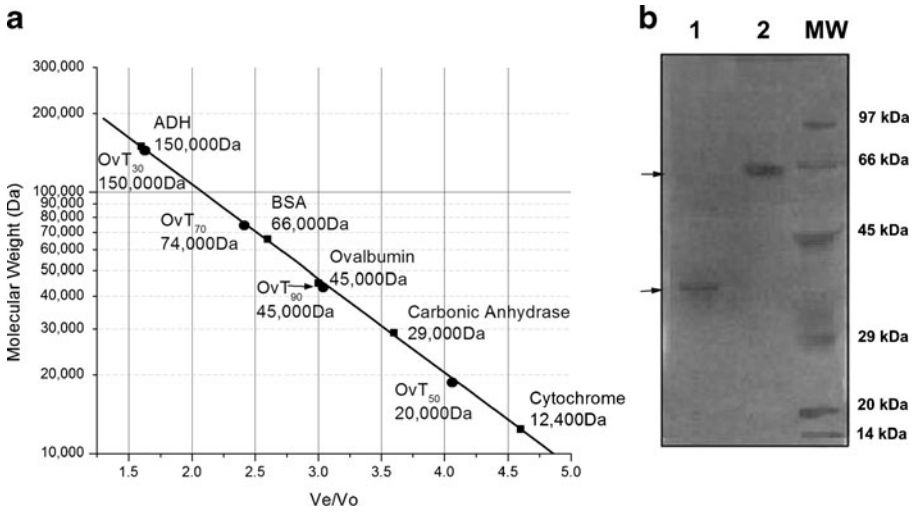


Fig. 3 **a** Plot of log molecular weight vs V_e/V_o of *Opuntia vulgaris* endo-1, 4-β-D-glucanase isoforms following gel permeation chromatography and **b** SDS–PAGE. *MW* protein molecular weight standard, *Lane 1* purified T_{90} isoform from endoglucanase, *Lane 2* purified T_{70} isoform from endoglucanase

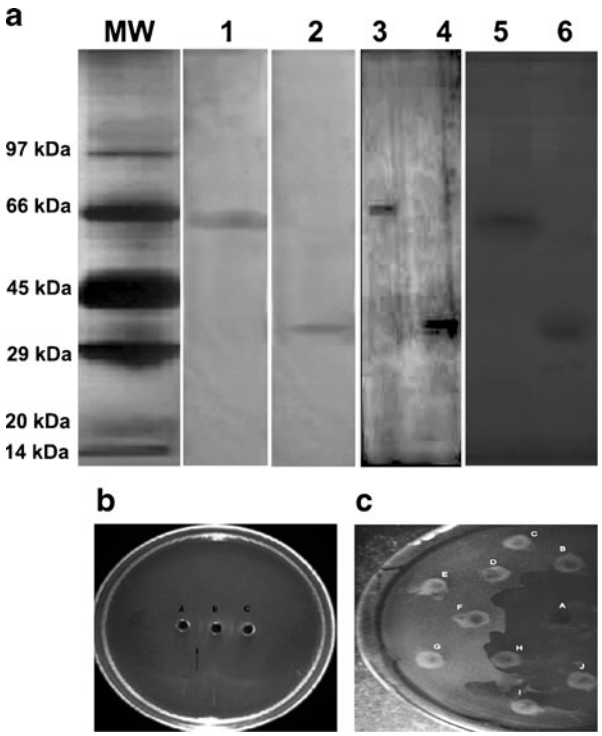


Fig. 4 **a** Western blot (*lanes 1* and *2*), native gel (*lanes 3* and *4*), and activity gel assay (*lanes 5* and *6*). *MW* protein molecular weight standard; *Lanes 1*, *3*, and *5* purified T_{70} isoform from endoglucanase; *Lane 2*, *4*, and *6* purified T_{90} isoform from endoglucanase. **b** Ouchterlony immunodiffusion of endoglucanase isoforms against rabbit polyclonal anti-endoglucanase antiserum. Clear precipitin lines are visible between *A*, *B*, and *C*. *A* and *C* purified OV T_{90} and OV T_{70} endoglucanase isoform; *B* polyclonal rabbit anti-*Opuntia vulgaris* endoglucanase T_{90} antiserum. **c** Congo red plate assay of purified T_{90} and T_{70} isoforms of *O. vulgaris* endoglucanase. (*A* distilled water as control; *B*, *C*, *D*, *E*, *F* purified OV T_{70} endoglucanase isoform; *G*, *H*, *I*, *J* purified OV T_{90}) endoglucanase isoform

(Fig. 4a) indicated the protein to be a monomer without disulphide bonds. The isolated cellulase protein samples of 0.4 U/mg, when run in the substrate incorporated gel, yielded bands with lytic activity as dark zones following UV illumination (Fig. 4a).

The K_m and K_m values were obtained (Table 2) from the Lineweaver–Burk double reciprocal plot. The endoglucanase T_{70} isoform had a K_m of 43 mM and an apparent V_{max} of 12.5 μmol min^{-1} μg^{-1} of protein, and the T_{90} isoform had a K_m of 40 mM and V_{max} of 10 μmol min^{-1} μg^{-1} of protein. The endoglucanase displayed a lower K_m for carboxymethyl cellulose, indicative of greater affinity for this substrate [27]. In the present work, the K_m value determined for achieving the half maximal reaction velocity employing carboxymethyl cellulose as substrate served as the reference. There is paucity of information on the K_m values for cellulose hydrolysis by cellulases. Values of 0.5 and 1.6 mg/ml have been reported in studies of carboxymethyl cellulose hydrolysis by cellulases of *Myrothecium verrucaria* and *T. reesei* [28, 29].

Table 2 Comparison of kinetic constants for endoglucanase isoforms

Substrate	Temp (°C)	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m
Opuntia vulgaris endoglucanase T_{70} isoform	70	43.4±0.1	12.5±0.07	0.28
Opuntia vulgaris endoglucanase T_{90} isoform	90	40.0±0.08	10.0±0.12	0.25

The K_m and V_{max} values were obtained from the Lineweaver–Burk double reciprocal plots of substrate saturation kinetics of the T_{70} and T_{90} *Opuntia vulgaris* isoforms

The generation of polyclonal rabbit antiserum against the T_{90} isoform of *O. vulgaris* endoglucanase aided to investigate cross-reactivity of the antiserum between the various endoglucanase isoforms. The existence of common epitopes in the polypeptide sequences of these different isoforms was thus recognized. Western blot analysis of endoglucanase isoforms showed cross-immunoreactivity between these polypeptide forms, although they were found differing in their optimum temperature. Western blot analysis (Fig. 4a) and Ouchterlony double immunodiffusion (Fig. 4b) yielded positive bands with polyclonal rabbit anti-endoglucanase antibody corresponding to the band position of T_{70} and T_{90} isoforms. This was further confirmed by the observation of the rabbit antiserum which brought about inhibition of the endoglucanase activities during in vitro test tube assays. The rabbit anti-endoglucanase antiserum was capable of inhibiting 85% of the endoglucanase activity of the T_{90} isoform, and 70% of the T_{70} isoform when assayed (Fig. 5). These observations confirmed that the polyclonal endoglucanase rabbit antibodies recognized both isoforms due to the existence of common epitopes displayed for antibody binding by these different isoforms.

Investigations carried out to determine a role for metal–protein interactions in the stabilization of thermophilic isoforms employed only the *O. vulgaris* T_{70} and T_{90} forms. The effect of divalent cations used at selective concentrations (1, 5, and 10 mM) in the assay cocktail followed by incubation of the metal–enzyme reaction mixture at 70 °C and at 90 °C (corresponding to the temperature optima of these isoforms) yielded endoglucanase activity as determined (Table 3). For the *O. vulgaris* T_{70} and T_{90} endoglucanase isoforms, maximum activity was observed with $Mn^{2+}>Co^{2+}>Cu^{2+}$. The metal ions Ca^{2+}, Zn^{2+} (1 mM each) enhanced about 98% and 71% of the *O. vulgaris* (T_{70} isoform), and more than 90% in the case of the T_{90} isoform. In the presence of 10 mM Mg^{2+}, nearly 90% of endoglucanase activity was retained by the T_{70} and T_{90} isoforms. Activity in the presence of 1 mM Ni^{2+} was approximately 70% and 60%, respectively, for both isoforms, and the presence of Fe^{2+}, EDTA, LiCl, and KCl contributed significantly to a reduction in the endoglucanase activity of both these thermophilic isoforms. Hg^{2+} completely abrogated the enzyme activity of both isoforms. Substrate specificity studies recognized remarkable activity of both isoforms towards carboxymethyl cellulose followed by cellobiose, chitin, and pectin, in that order (Fig. 6), confirming the fact that the plant enzyme under investigation was of the endoglucanase type.

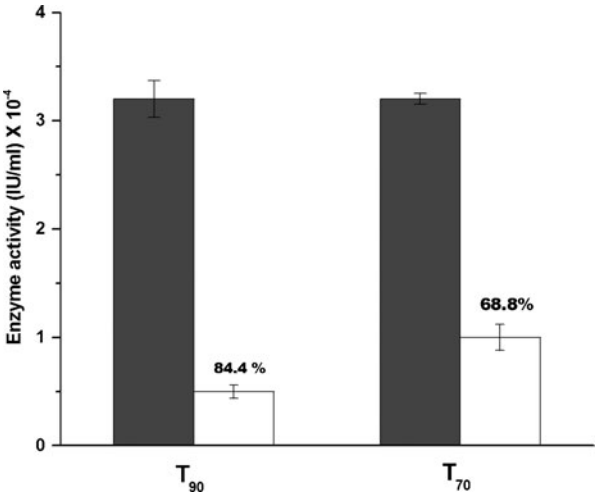


Fig. 5 Inhibition assay of *Opuntia vulgaris* endo-1, 4-β-D-glucanase isoforms using rabbit polyclonal anti-endoglucanase T_{90} antiserum. *Values* are the mean of three independent assays. **a** Control enzyme (*black bar*), **b** Control enzyme + Ab treated (*white bar*)

Table 3 Effect of cations on endoglucanase T_{70} and T_{90} isoforms

Opuntia vulgaris endo-1,4-β-D-glucanase T_{70} and T_{90} isoform activity (%)						
Sample	1 mM		5 mM		10 mM	
Control cellulase	100	100	100	100	100	100
Cellulase + $MnCl_2$	264	317	244	240	232	219
Cellulase + $CoCl_2$	164	143	144	112	140	93
Cellulase + $CuCl_2$	113	122	98	95	89	67
Cellulase + $CaCl_2$	98	99	84	85	75	79
Cellulase + $ZnCl_2$	71	94	67	88	48	24
Cellulase + $NiCl_2$	73	56	64	42	50	22
Cellulase + $MgCl_2$	54	42	72	67	98	89
Cellulase + $FeCl_2$	61	26	54	12	20	7
Cellulase + LiCl	45	14	31	9	12	5
Cellulase + EDTA	24	13	14	6	3	9
Cellulase + KCl	18	4	11	2	9	1
Cellulase + $HgCl_2$	0	0	0	0	0	0

The enzymes were treated with EDTA, and the activities were determined as described in the "Materials and Methods" Section. These data correspond to an experiment representing a total of three independent experiments closely coincident

Conclusions

Research on cellulases has progressed very rapidly over the past five decades. The availability of xerophytic thermophilic enzymes from plant species for use of high-temperature industrial processing needs remains a viable option for their use where thermophilic enzymes were required. The xerophytic endoglucanase showed the existence of multiple forms, enhanced thermostability, and pH stability of these enzyme activities

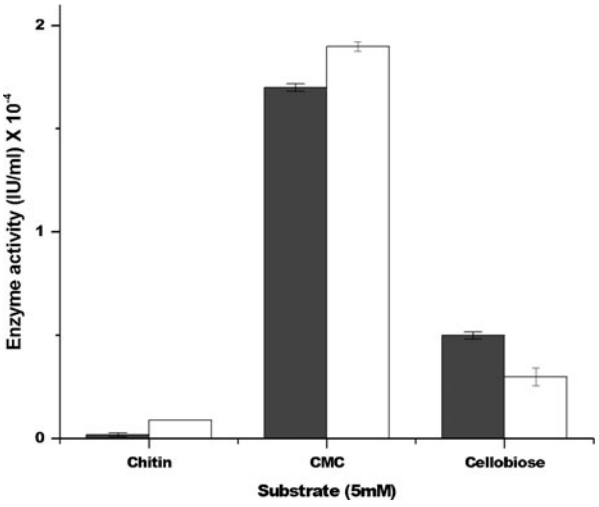


Fig. 6 *Opuntia vulgaris* T_{70} and T_{90} endo-1, 4-β-D-glucanase isoform substrate specificity. *Values* are the mean of three independent assays. The T_{70} and T_{90} isoforms of *O. vulgaris* (0.1 mg/ml) were assayed using each (5 mM) substrate (pectin, chitin, carboxymethyl cellulose, cellobiose) at 70 °C and 90 °C (T_{70} isoform (*black bar*), T_{90} isoform (*white bar*). Both isoforms exhibited remarkable activity towards carboxymethyl cellulose establishing endoglucanase activity

under acidic as well as under alkaline conditions. Thermostable enzymes tolerate higher temperatures, and gave a longer half-life to the catalytic function and inhibited microbial growth, decreasing the possibility of microbial contamination. Their potential for degradation of complex carbohydrates at elevated temperatures allows us to generate strategic opportunities for use of thermophilic enzymes in industrial needs.

Novelty Statement

The novelty of the present work is the discovery of a thermostable enzyme in eukaryotic origin. No hyperthermophilic in eukaryotic origin has been reported till date. The availability of novel Endo 1, 4 β-D-glucanase from *O. vulgaris* can withstand 90 °C and also withstand both alkaline and acidic pH; therefore, it has great potential for industrial applications, including the production of biofuels.

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