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Modification of guar galactomannan with the aid of *Aspergillus niger* pectinase

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Abstract

Pectinase from *Aspergillus niger* associated with polygalacturonase activity, caused considerable debranching–depolymerization of guar galactomannan. Zymogram analysis further confirmed the action of pectinase on the latter. Optimum activity occurred at pH 5.0 and 50 °C and the reaction obeyed Michaelis-Menten kinetics with K_m and V_{max} values of 3.72 mg mL^{-1} and 1.852 mmoles min^{-1} mg^{-1}. Viscometry, GPC and HPLC analyses of the native guar galactomannan showed a molecular weight (M_w) of ~240 kDa with a galactose:mannose (G:M) ratio of 1:1.6, as revealed by GLC analysis, whereas, after enzymatic catalysis for 60 min, the major resultant product had a M_w of ~70 kDa with a G:M ratio of 1:2.8, respectively. The latter had a solubility of ~98% in water at ambient temperature (within 30 min) compared to ~60% for native galactomannan. IR spectral analysis revealed differences in the conformation of enzyme catalyzed guar galactomannan. The latter showed better gelling property with xanthan gum compared to native galactomannan. Use of non-specific pectinase for debranching–depolymerization of guar galactomannan is of commercial interest as modified galactomannans find multiple applications as functional food ingredients.

Keywords: Guar; Galactomannan; Pecinase; Debranching–depolymerization; Rheology

1. Introduction

Galactomannans derived from guar, tara and locust been gums are finding wide spread applications based on their ability to thicken and stabilize many food products, as functional food ingredients and also as a source of dietary fiber (Stephen & Churms, 1995; Greenberg & Slavin, 2003). Guar galactomannan is a natural water-soluble polysaccharide obtained from the seed endosperm. Structurally, it contains a linear backbone of 1→4-linked β-D-mannose units to which there is an attachment of single α-D-galactose residue at C6 of mannose with 1→6 glycosidic bonds, so as to have a galactose:mannose ratio of ~1:1.6–2.0, respectively. Guar galactomannan is the material of choice in various industries such as food, oil recovery, cosmetics, pharmaceuticals, textile, etc. owing to its easy availability at low cost and excellent viscosity properties. However, modification (including debranching/depolymerization) of guar galactomannan is beneficial for its diversified applications. Depolymerized guar galactomannan with relatively low M_w and low viscosity is of use as a source of soluble dietary fiber in nutraceuticals products and functional foods. In comparison with the conspicuous thixotropic property of native guar gum solutions, the partially hydrolyzed guar gums with lower M_w are easily soluble in water with better clarity of solution (Greenberg & Slavin, 2003).

Depolymerization of guar galactomannan could be achieved either by controlled acid hydrolysis or using specific enzymes such as mannosidase and/or galactosidase; in the former the reaction proceeds randomly, sometimes giving a large excess of free monomers, whereas in the latter the reaction can be kinetically controlled with desirable end products. Nevertheless, these enzymes are generally expensive, and are not successful in partially debranching guar galactomannan to a product mimicking G:M ratio of

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locust bean gum (G:M of 1:4). Nevertheless, several studies on non-specific enzyme depolymerization of carbohydrate macromolecules have earlier been carried out successfully (Pantaleon, Yalpani, & Scollar, 1992; Kittur, Vishu Kumar, Gowda, & Tharanathan, 2003; Vishu Kumar, Gowda, & Tharanathan, 2004). Such an approach is preferred alternative route for expensive enzymes and for value addition. In the present study, commercially available-inexpensive pectinase from *Aspergillus niger* was made use of in partial debranching of guar galactomannan. A kinetic study of optimum depolymerization of guar gum by pectinase has been determined, which is of use in the partial depolymerization process so as to get products with water solubility and enchanced G:M ratio. In addition, the gelling properties of both native and modified guar galactomannans were determined.

2. Materials and methods

2.1. Materials

Guar galactomannan was a commercial food grade sample purchased from Aqualon, Wilmington, DE, USA. Standard sugars (mannose and galactose), sodium borohydride, pectinase (from *A. niger*, EC 3.2 1.15), Sepharose CL-2B and pectin (citrus) were obtained from Sigma Chemical Co., St Louis, MO, USA. Other chemicals used were of highest purity available.

2.2. Methods

2.2.1. Guar galactomannan characterization—determination of molecular weight (M_w)

(a) *By viscometry*. The relative viscosity of galactomannan dissolved in distilled water (0.1–0.5%) was determined using Ostwald's viscometer. The average M_w was calculated using Mark-Houwink's equation, $\eta = K(\text{molecular mass})^{\alpha}$, where η = intrinsic viscosity, $k = 3.04 \times 10^4$ and $\alpha = 0.747$ (Cheng, Brown, & Prudhome, 2002).

(b) *By gel permeation chromatography (GPC)*. The galactomannan solution (1 mL of 0.5% in water) was loaded onto Sepharose CL-2B column (bed volume, 140 mL) and eluted with water. Fractions (1.5 mL) were analyzed for total sugar by phenol-sulphuric acid method (Rao & Pattabiraman, 1989). The column was pre-calibrated with dextran T-series standards.

(c) *By high-performance size exclusion chromatography (HPSEC)*. HPSEC was performed on Shimadzu LC 8A system connected to RI detector, using E-linear and E-1000 columns (Waters Associates, Millford, USA) connected in series. Distilled water was used as the mobile phase at a flow rate of 0.6 mL/min. The column was pre-calibrated with dextran T-series of known M_w.

2.2.2. Pectinase assay

To 0.9 mL sodium pectate (0.25% of pectic acid in 0.1 M sodium citrate buffer of pH 3.0, w/v) was added 0.1 mL of *A. niger* pectinase (60 µg), incubated at 25 °C for 30 min. The reaction was stopped by adding 1.0 mL dinitrosalicylic acid reagent to the reaction mixture and keeping in a boiling water bath for 15 min. After cooling, 3.0 mL double distilled water was added and the absorbance was measured at 530 nm (Miller, 1959). One unit of pectinase activity is defined as the amount of enzyme required to liberate 1 µmole reducing sugar per min at 25 °C.

2.2.3. Pectinase assay using guar galactomannan as the substrate

To 1.0 mL 0.5% (w/v) guar galactomannan solution was added 25 µL of 0.1% (w/v) *A. niger* pectinase, incubated at ambient temperature for 30 min followed by arresting the reaction by the addition of 2 vol. of absolute ethanol. The contents were mixed thoroughly and centrifuged (4000 rpm for 10 min). The reducing sugar released into the supernatant was assayed by modified Schale's method (Imoto & Yagishita, 1971) and the precipitate was analyzed for sugar composition and M_w. The enzyme activity was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute at ambient temperature.

2.2.4. Polyacrylamide gel electrophoresis (PAGE) and zymogram analysis

PAGE analysis was performed on 10% gel according to the method of Laemmli (1970) and the isolated protein bands were visualized with Coomassie Brilliant Blue. For zymogram analyses, 0.1% (w/v) of citrus pectin and guar galactomannan were incorporated separately into the gel followed by protein separation. After the run, for pectinase activity, the gel was incubated at pH 3.5 for 1 h, whereas for galactomannanase activity, the gel was incubated at pH 5.0 and 50 °C for 1 h, after which the gels were stained using Ruthenium red and Congo red, respectively. For intensification of the latter stain, 5% acetic acid was added (Kittur et al., 2003).

2.2.5. Enzyme kinetics

Optimal enzyme concentration was determined by the addition of 10–50 µg of pectinase to 0.5% (w/v) guar galactomannan solution (1 mL) and assaying the reaction. The effect of pH in the range 3.0–9.0 was determined by the addition of 0.1 N HCl or 0.1 N NaOH to the galactomannan solution, whereas the effect of temperature was studied between 24 and 60 °C using galactomannan solutions at optimal pH. By varying the substrate concentration between 1 and 10 mg mL^{-1}, the effect of substrate concentration on pectinase activity was determined, whereas the apparent K_m and V_{max} were calculated from the double reciprocal plot. The optimum pH and temperature stability of pectinase was studied by pre-incubating the enzyme at different pHs and temperatures followed by enzyme assay.

2.2.6. Isolation and characterization of depolymerization products

The precipitate obtained from above was repeatedly washed with alcohol and lyophilized. The supernatant was concentrated further by flash treatment. It was characterized for M_w by viscometry, GPC and HPLC and its galactomannan content was determined by gas liquid chromatography after hydrolysis and conversion into alditol acetate derivatives. Briefly, 10–15 mg samples were suspended in 0.28 mL water and allowed to swell for 2–3 h followed by the addition of 0.72 mL conc. H_2SO_4 at cold temperature. Concentration of H_2SO_4 was brought to ~5.5% (v/v) by the addition of 12 mL water and the mixture was kept in a boiling water bath for 10 h. After cooling, the acid was neutralized by the addition of solid barium carbonate and the resulting mixture was filtered. The filtrate was deionized with Amberlite IR 120 H^+, concentrated by rotary flash evaporation and reduced with sodium borohydride. The purified alditols were acetylated by heating (100 °C/1 h) with acetic anhydride and pyridine (0.5 mL each) and analyzed by GLC on OV-225 column (1/8 in.×6 ft ss column) connected to a Shimadzu gas liquid chromatograph equipped with flame ionization detector at 200 °C (Sawardekar, Sloneker, & Jeanes, 1965).

2.2.7. IR spectroscopy

IR-spectral studies were performed on Perkin–Elmer 2000 spectrometer under dry air at room temperature using KBr pellets. 4 mg of the samples were mixed thoroughly with 200 mg solid KBr from which 40 mg were taken for pelletization, spectra were taken between 4000 and 400 cm^{-1} and the reproducibility of the data was verified on two preparations.

2.2.8. Gel strength determination

The force–distance curve of partially depolymerized guar gum samples (100 mg) with xanthan gum (100 mg) was measured by mixing them in a ratio of 1:1 to get a final concentration of 0.8% (w/v). The mixture was kept in the cold (5 °C) overnight and the resulting gel was compressed (75%) using a probe of 100 mm diameter and cross-head speed of 100 mm per min.

3. Results and discussion

3.1. Characterization of guar galactomannan

The average M_w of native guar galactomannan as determined by viscometry was ~240 kDa, which was in agreement with those determined by GPC and HPSEC. The appearance of a single peak in HPSEC further confirmed its homogeneity (Fig. 1A). The galactose: mannose ratio of the latter was 1.0:1.6 as determined by GLC and the viscosity of its aqueous solution (1%) was ~5100 cps.

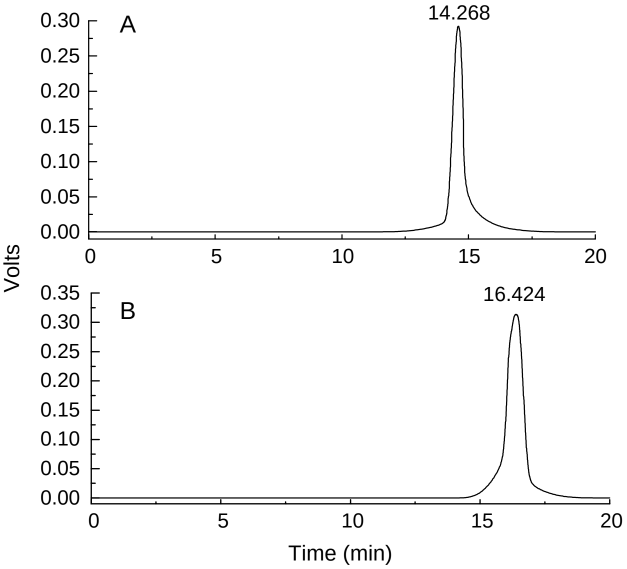

Fig. 1. HPSEC profiles of (A) native and (B) pectinase depolymerized guar galactomannan (reaction time, 1 h).

3.2. Enzyme activity

During screening experiments with various non-specific enzymes such as papain (papaya latex), pepsin (porcine stomach mucosa), hemicellulase (*A. niger*), pronase (*Streptomyces griseus*) and pectinase (*A. niger*), we observed association of guar galactomannan depolymerizing/debranching effect exclusively with pectinase from *A. niger*, whereas the other enzymes screened did not show any such activities. Commercial *A. niger* pectinase showed a specific activity of 9.85 U mg^{-1} towards pectin, whereas its non-specific activity towards guar galactomannan was 3.01 U mg^{-1}, indicating its preference to specific substrate. Upon native-PAGE, the enzyme was resolved into four distinct bands (Fig. 2A) whose zymogram analysis

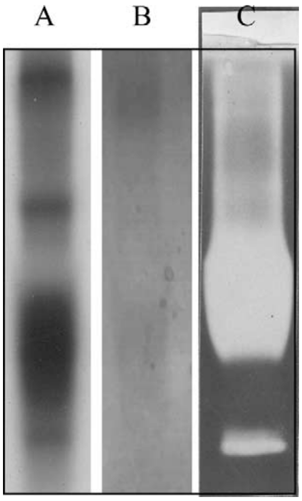

Fig. 2. Polyacrylamide gel electrophoregram of commercial pectinase (A) and zymogram analyses of galactomannanase (B) and pectinase (C) activities.

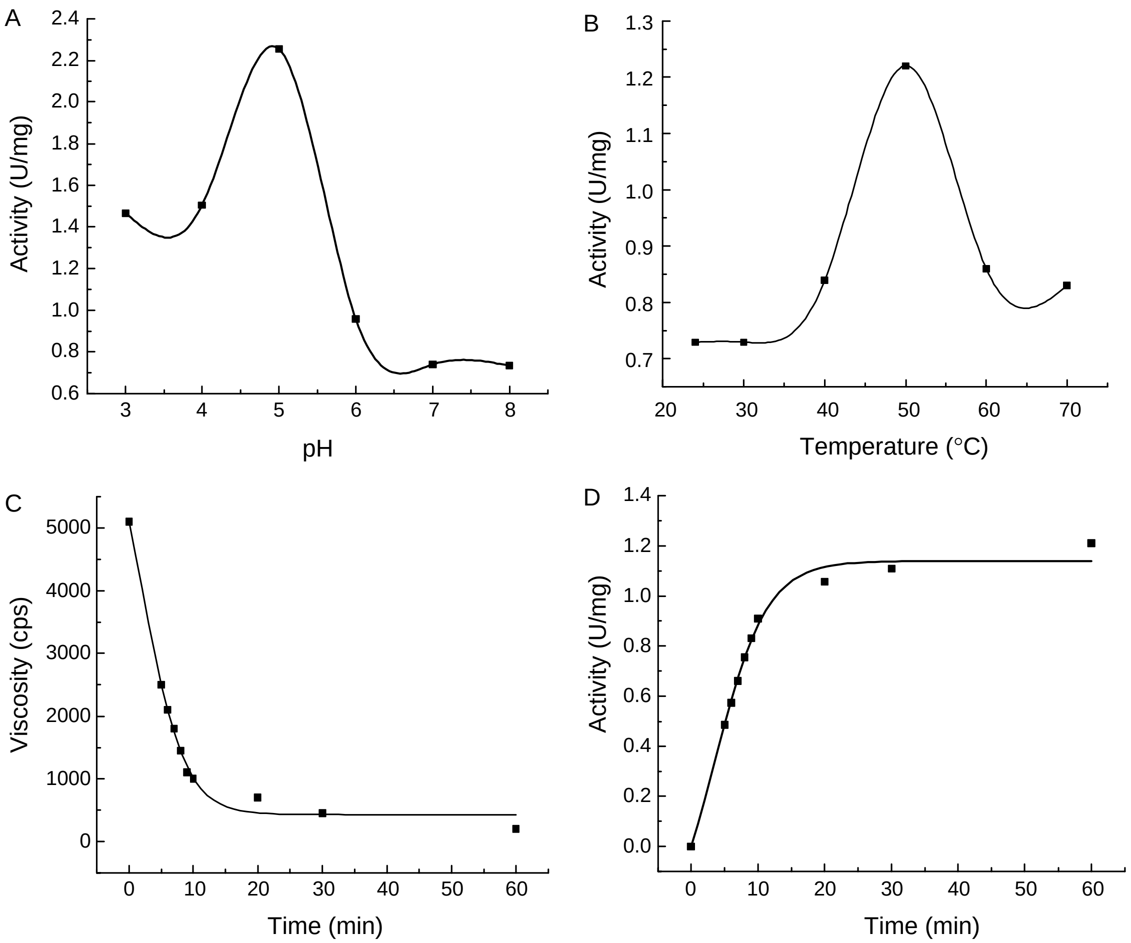

Fig. 3. Kinetics of pectinase towards galactomannanase activity, (A) effect of pH, (B) effect of temperature, (C) and (D) guar solution viscosity and reducing equivalents released during the course of reaction.

using specific substrate (i.e. citrus pectin) showed association of pecinase activity with all the bands (Fig. 2B), indicating them to be isozymes. Towards guar galactomannan also, all the isozymes showed depolymerizing/debranching action as could be seen from Fig. 2C.

A gradual decrease in the viscosity of guar galactomannan during the course of time was indicative of endo-action of the isozymes, whereas release of galactose and mannose into the supernatant obtained after arresting enzymatic reaction, as revealed by HPLC profile and TLC was indicative of their exo-action. The latter was further confirmed by increase in reducing sugar content in the supernatant along with the reaction time. The presence of both galactose and mannose was indicative of debranching as well as depolymerizing action of the isozymes, confirming enzyme non-specificity. Optimum depolymerization/debranching occurred within 120 min after which there was a gradual decrease in the activity.

For further assay, the commercial pectinase without further purification was used and the effect of pH and temperature during its action on guar galactomannan is represented in Fig. 3A, B. The effect of enzyme

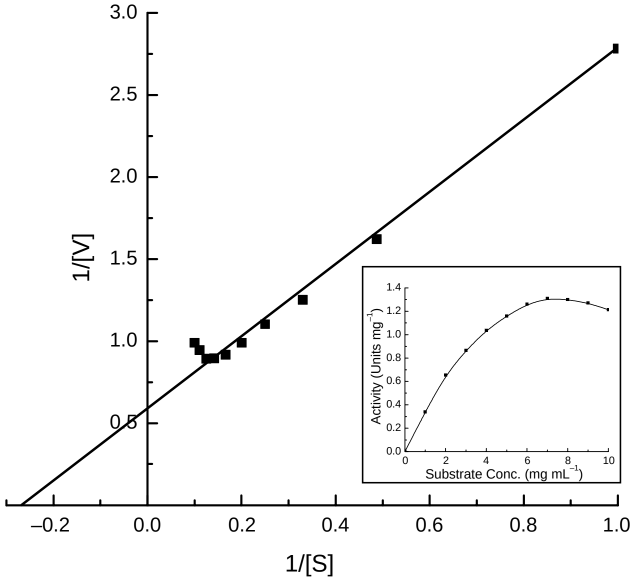

Fig. 4. Double reciprocal plot (Inset: effect of guar galactomannan concentration on pectinase activity).

Table 1
Depolymerization of guar galactomannan by *Aspergillus niger* pectinase

Sample	Depolymerization time (min)	M_w (kDa)	G:M ratio
Guar galactomamman	0	240	1:1.6
Depolymerized galactomannan	15	150	1:2.1
	30	100	1:2.3
	45	90	1:2.5
	60	70	1:2.8

concentration followed first order reaction kinetics up to a concentration of 100 μg, beyond which they were of second and zero orders and hence for further study, an enzyme: substrate ratio of 1:100 was used. The optimum galactomannanase activity occurred at pH 5.0 and 50 °C, whereas pectinase activity occurred at pH 3.5 and 25 °C. Such differences could be due to subtle pH-dependent conformational changes during its dual activities (Vishu Kumar & Tharanathan, 2004).

Fig. 3C, D represent effect of reaction time on gum solution viscosity and reducing equivalents released. As it is evident from the graphs, 70% reduction in viscosity with release of ~70 reducing equivalents occurred within 10 min indicating the predominance of endo-action (i.e. depolymerizing) of the enzyme followed by its debranching effect. This was also confirmed by TLC analysis, wherein the supernatant obtained in the initial time showed only a trace amount of mannose whereas the 60 min supernatant showed the presence of both mannose and galactose.

The effect of substrate concentration showed a hyperbolic curve, which was indicative of Michaelis-Menten kinetics. The K_m and V_{max} calculated from the double reciprocal plot were 3.72 mg mL^{-1} and 1.852 mmoles min^{-1} mg^{-1}, respectively (Fig. 4). A decrease in the activity beyond certain concentration of substrate was indicative of product or substrate inhibition. The latter was ruled out by incubation of galactose/mannose with the enzyme for 15 min prior to the assay. The phenomenon of substrate inhibition can be rationalized by assuming that a second substrate molecule binds non-productively to the active site thereby inhibiting the productively bound one (Sannchez-Ferres, Franscisco, & Franscisco, 1993).

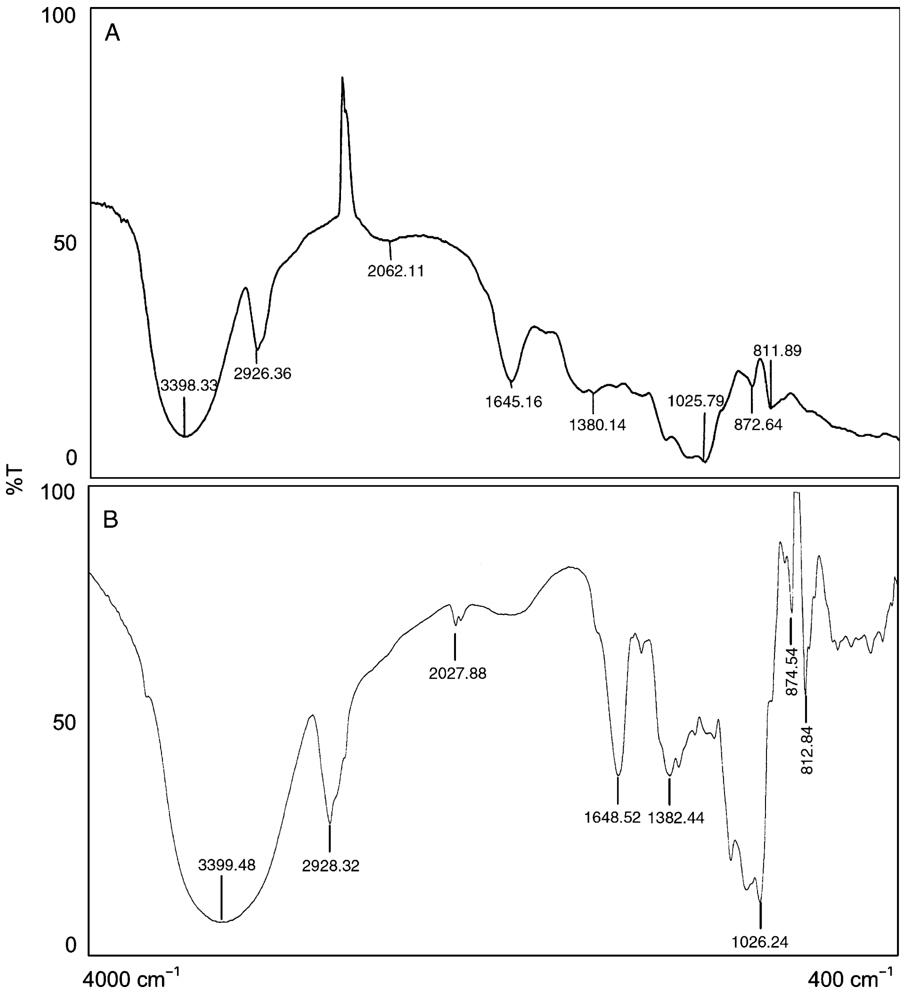

Fig. 5. FT-IR spectra of native (A) and pectinase treated (B) guar galactomannan.

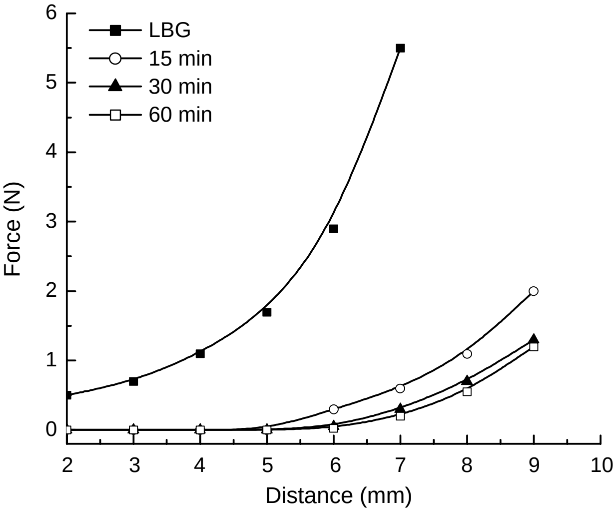

Fig. 6. Force–distance curves of LBG and 15–60 min pectinase depolymerized guar galactomannans with xanthan gum.

Pre-incubation of pectinase at different pH (pH stability study) indicated that the enzyme is stable at pH 5.0 for 2 h. The temperature stability study indicated that the enzyme was stable up to 37 °C, beyond which there was a decline in the activity and at 50 °C the activity was stable only for 30 min and after 2 h there was 50% reduction in the activity. So for depolymerization/debranching activity study, a substrate solution pH of 5.0 and ambient temperature (∼25 °C) were chosen.

3.3. Characterization of the products

There was a change in the M_w of depolymerized guar galactomannan with reaction time (Table 1), as also could be seen from the change in viscosity (Fig. 3C). Appearance of a single peak was indicative of molecular homogeneity of the product obtained (Fig. 1B, product obtained after 1 h reaction time), which could be due to the splitting action of enzyme at regular intervals. There was a gradual change in the G:M ratio also (Table 1). The dual action (i.e. debranching as well as depolymerizing) of pectinase was further confirmed by TLC analysis of the supernatant, which showed the presence of both galactose and mannose (profile not shown) and the latter further confirmed the exo-action of pectinase. The native galactomannan had a water solubility of 62%, whereas the enzyme modified products were readily soluble in water.

In the FT-IR spectra (Fig. 5), the region between 3000 and 2800 cm^{-1} represents—C–H stretching modes (Kacurakova, Belton, Wilson, Hirsch, & Eblingerova, 1998) and that around 3300 cm^{-1} was due to hydroxyl stretching vibration of polysaccharides and water involved in hydrogen bonding (Fringant, Tvaroska, Mazeau, Rinaudo, & Desbrieres, 1995). The gradual appearance of a shoulder in the –CH stretching region (3000–2800 cm^{-1}) of the spectra following enzyme action was due to the galactosidase-type action of pectinase along with depolymerization as a result of exposure of the C6. Similarly, a marked modification of the shape and intensity of –CH$_2$ (∼2930 cm^{-1}) stretching band suggests different arrangement of the native and depolymerized gums, which also includes decrease in the molecular size. The other main features observed were the spectral region between 800 and 1200 cm^{-1}, which is due to highly coupled C–C–O, C–OH and C–O–C stretching modes of polymer backbone (Kacurakova et al., 1998). The absorption band around 1648 cm^{-1} due to associated water and the region around 1430–1400 cm^{-1} due to—CH$_2$ deformation modes were also seen (Kacurakova, Ebringerova, Hirsch, & Hromadkova, 1994). A sharpening of the absorption around 1646 cm^{-1} in the depolymerized product was indicative of its increased association with water, which in turn explains its improved solubility compared to native guar. The region between 700 and 500 cm^{-1} is thought to be sensitive to change in crystallinity (Tul'Chinsky, Zurabyan, Asankozhoev, Kogan, & Ya, 1976) and the ratio of $A_{1423\ \mathrm{cm^{-1}}}/A_{823\ \mathrm{cm^{-1}}}$ is indicative of crystallinity index (CrI) (Nelson & O'Connor, 1964). A change in the spectra in this region was indicative of conformational changes and the CrI calculated for native and depolymerized guar galactomannan were 0.24 and 0.32, respectively, indicating more crystallinity of the product, which could be possible because of its smaller size.

The pectinase modified gum samples were further characterized for their gelling properties. The enzyme-treated gums were easily soluble in cold water unlike locust bean gum (LBG, used in most of the gels along with xanthan gum), which required hot water for complete dissolution. Gel strength experiments were performed to determine the rupture in the force–distance curve (Fig. 6), in comparison with LBG+xanthan gum, which was used as the control. The data (Table 2) revealed the formation of a very soft gel without any rupture point, whereas

Table 2
Determination of gel strength of pectinase–depolymerized guar gum samples

Substrate (1%, mL)	Enzyme (μg)	Time (min)	G-M ratio	Viscosity (cps)	Distance (mm)	Force (N)
100	300	15	1:2.1	∼800	9.05	1.91
100	300	30	1:2.3	∼620	9.0	1.18
100	300	45	1:2.5	∼540	8.97	1.19
100	300	60	1:2.8	∼400	8.5	0.59
100	300	70	1:2.9	∼280	9.01	0.79

LBG-xanthan gum gel (Fig. 6) was much stronger. Once the compression force was removed from the former gel, it partially regained the original status.

In conclusion, partial depolymerization of guar gum could be achieved by *A. niger* pectinase, which being inexpensive and food grade is of commercial importance and utility. The partially hydrolyzed guar galactomannans, in addition to decrease in M_w/viscosity underwent modification in the G:M ratio. Because of its easy solubility in aqueous medium and its soft gelling property, the depolymerized products may be of value as functional food ingredients.

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