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Nonspecific Activity of *Bacillus acidopullulyticus* Pullulanase on Debranching of Guar Galactomannan

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Pullulanase (EC 3.2.1.41) from *Bacillus acidopullulyticus* could preferentially remove galactose residues from guar galactomannan (GG), a heteropolymer consisting of polymannan backbone with alternating galactose as single-residue side-chain stubs. The enzyme showed maximum activity at pH 4.5 and 45 °C and exhibited Michaelis−Menten kinetics with a K_m and V_{max} of 6.0 mg mL^{-1} and 1820 nmol min^{-1} mg^{-1}, respectively. The native and modified GG had a viscosity of 4800 and 3800 cps, respectively. The molecular mass of the enzyme-treated GG varied between 152 ± 0.5 kDa, and its GLC analysis revealed a significant change in galactose−mannose content of 1:3.8, respectively, similar to that of locust bean gum (LBG). FTIR and solid-state CP-MASS ^{13}C NMR analyses indicated subtle changes in the conformation of modified GG because of the removal of galactose residues. Gel-strength measurements with κ-carrageenan showed an improvement in the gelling behavior, similar to that shown by LBG. Debranching of GG by pullulanase is an alternative and inexpensive route to produce modified GG with enhanced functional properties, as a cost-effective replacement for LBG.

KEYWORDS: Guar galactomannan; pullulanase; debranching; locust bean gum; κ-carrageenan

INTRODUCTION

Guar galactomannan (GG), a natural polysaccharide derived from the seeds of the endospermic leguminous plant *Cyamopsis tetragonalobus*, is one of the most commercially exploited hydrocolloids. GG finds numerous applications in food and nonfood industries, viz. pharmaceutical, textile, oil recovery, mining, personal care, etc., because of its excellent viscosifying properties, easy availability, and low cost (*1−3*). GG is a copolymer of β-1,4-D-mannopyranose as backbone residues attached with α-1,6-D-galactopyranose as single-residue side-chain stubs. Locust bean gum (LBG), another functionally useful hydrocolloid, is also a galactomannan, having side-chain galactose stubs on every fourth mannose residue. It shows better gelling properties than GG, which is of high functional value. Thus, the distribution of galactose residues, either in uniform or block-wise pattern, on the mannan backbone plays a vital role in its functional property (*4, 5*). Hence, a selective removal of galactose residues, similar to LBG type, without significant damage to mannan backbone leads to improvement in the use of GG.

Debranching of GG has been efficiently achieved by enzymatic hydrolysis with α-galactosidase obtained from various sources (*6*). McCleary (*7*) and Bulpin et al. (*8*) employed α-galactosidase purified from coffee bean and guar legume for depletion of galactose residues from GG, which showed enhanced functional properties. A report from Overbeeke et al. (*9*) describes the expression of α-galactosidase from guar plant by transformation using recombinant DNA technology to produce galactose-depleted GG. Although successful, this technology is limited because only the α-galactosidase isolated from germinated guar seeds is highly effective in the removal of galactose residues. Also, the high cost involved for the isolation of this enzyme and its laborious production steps are non-economical for bulk use (*10*).

Nevertheless, recent literature reveals the nonspecificity of various enzymes belonging to hydrolases and rules out the concept of one enzyme to one substrate or group of related substrates (*11*). Nonspecific action of β-1,4-glycanase on mannans and cellobiose (*12*), papain (*13*), pronase (*14*), pepsin (*15*), and wheat germ lipase (*16*) on chitosan has been reported. The present study describes the nonspecificity of pullulanase (from *Bacillus acidopullulyticus*) in the selective removal of galactose residues from GG for improvement in its functional properties (gelling behavior) by facilitating synergistic interaction with other polysaccharides. As a result, the thickening property of GG becomes transformed into gelling property in the modified GG. This property is exploited in gelling applications, where specific gel and textural characteristics are required.

MATERIALS AND METHODS

Pullulanase (from *B. acidopullulyticus*, 400 units), Sephadex G-200, and Sepharose CL-4B were obtained from Sigma Chemicals (St. Louis, MO). Guar gum was procured from Hindustan Gum Chemicals, Haryana, India. Other chemicals used were of the highest purity available.

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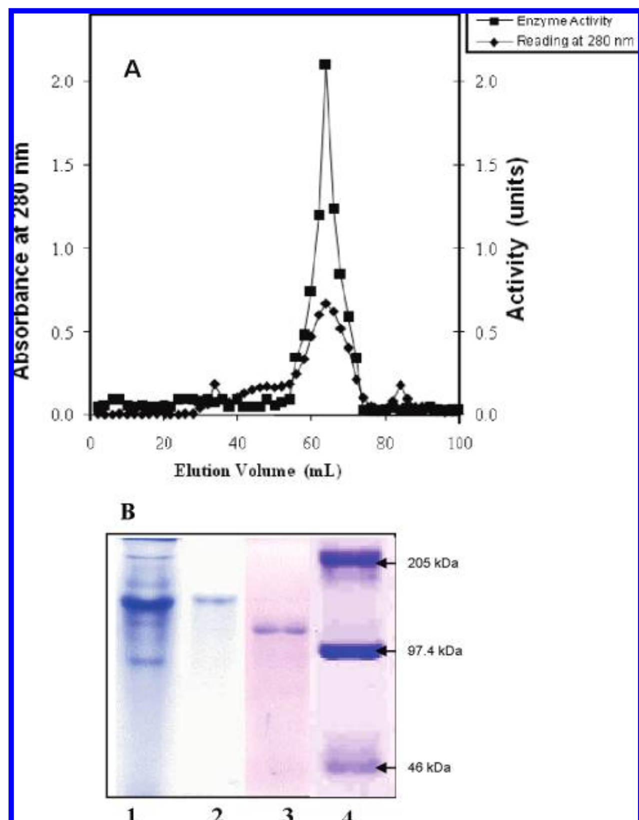

Figure 1. (A) GPC profile of pullulanase. (B) Native PAGE: lane 1, crude enzyme; lane 2, purified enzyme; lane 3, SDS−PAGE purified enzyme; lane 4, protein markers.

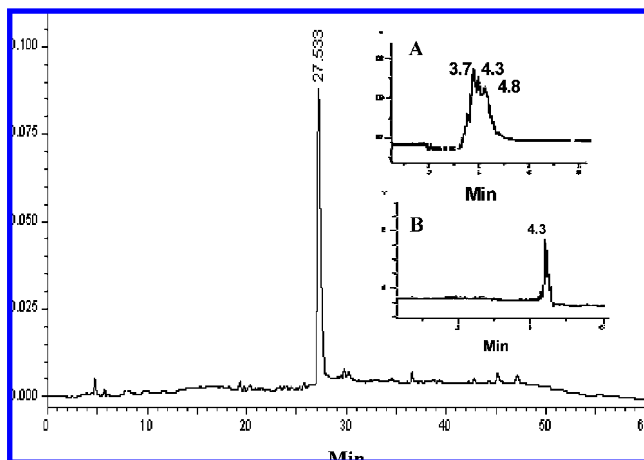

Figure 2. RP-HPLC profile of purified pullulanase. (Inset) Capillary electropherogram of (A) native and (B) purified enzyme.

Criteria of Enzyme Purity. *Gel-Permeation Chromatography (GPC).* The crude enzyme solution was loaded onto Sephadex G-200 column (100 cm length × 0.7 cm inner diameter) and eluted with 50 mM phosphate buffer at pH 7.4. Fractions were analyzed at 280 nm, and the one that showed catalytic activity towards both pullulan and GG was collected.

Native/Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Native and SDS−PAGE were performed on 7.5% gel according to the method of Laemmeli (*17*), and the isolated protein bands were visualized by staining with Coomassie Brilliant Blue.

Capillary Zone Electrophoresis (CZC). CZC was performed on a Prince 550 system (from The Netherlands) with a fused silica capillary column connected to a UV detector at 10 kV, 100 mbar, using Tris glycine buffer (pH 8.8). The enzyme solution (stock of 10 mg mL^{-1}, ~10 nL) was injected under a low-pressure hydrodynamic injection time of 2 s. Data acquisition was performed using DAx software.

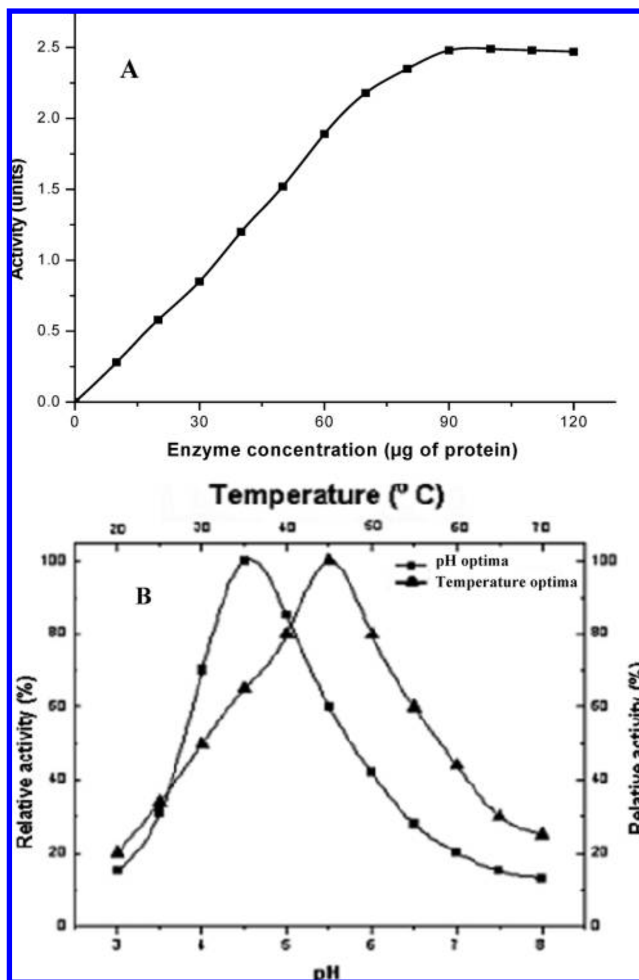

Figure 3. Kinetic study of pullulanase toward GG: (A) effect of the enzyme concentration and (B) pH and temperature optima.

Table 1. Kinetic Parameters of Pullulanase towards Specific and Nonspecific Substrates

substrate	activity (units)	optimum pH	optimum temperature (°C)
pullulan	15.2	5.5	50
guar galactomannan	2.2	4.5	45

Reverse-Phase High-Performance Chromatography (RP-HPLC). RP-HPLC was performed on Shimadzu LC-8A system connected with a C-18 (ODS) column. A total of 20 μL of sample was injected into the column. The mobile phase used was solvent A (0.1% trifluroacetic acid, TFA) followed by a gradient run consisting of 0% solvent B (70:30 acetonitrile/water with 0.05% TFA) traversing to 100% in 90 min at a flow rate of 1.0 mL min^{-1}. The elution was monitored at both 230 and 280 nm.

Enzyme Activity. The enzyme activity was determined by co-incubation of pullulan (1%) and pullulanase (10 μg, 1 mL) at pH 5.0 for about 1 h at 37 °C and estimating the reducing sugar released by the DNS method (*18*). The specific activity was expressed as μmol of reducing equivalents released min^{-1} (mg of protein)$^{-1}$.

Activity toward GG (0.5% solution) was determined by incubating with the enzyme (70 μg, 1 mL) for about 1 h at 37 °C. The reaction was terminated by the addition of 3 vol of ethanol followed by centrifugation. The supernatant was assayed for the reducing sugar released by the ferricyanide method (*19*), and the activity (unit) was expressed as μmol of reducing equivalents released min^{-1} (mg of protein)$^{-1}$.

Kinetic Study. The optimum pH for selective debranching of GG was studied at pH between 3 and 9, and the temperature optimum was determined by incubation at temperatures between 25 and 70 °C. The Michaelis−Menten constants K_m and V_{max} were evaluated from

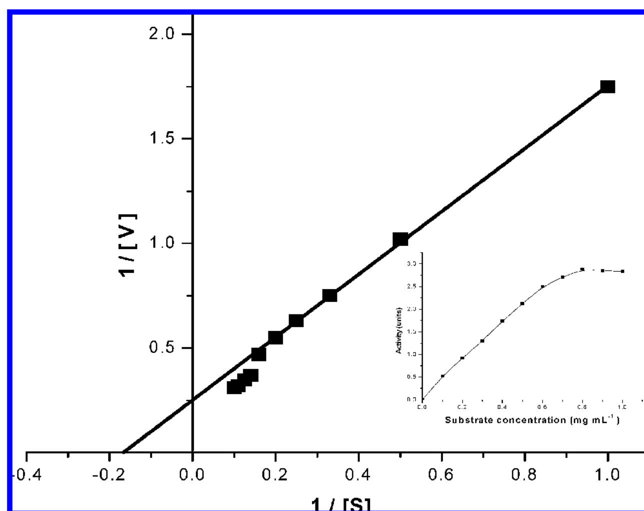

Figure 4. Double-reciprocal plot. (Inset) Effect of the substrate concentration on enzyme activity.

Lineweaver−Burg (double-reciprocal) plot of initial velocity versus substrate concentration. pH and temperature stabilities were determined by pre-incubation of the enzyme at different pH values and temperatures followed by measuring the residual activity.

Isolation of Partially Hydrolyzed GG (PHGG). GG solution (0.5%) together with pullulanase in the ratio of 70:1 (w/w) was incubated for different periods (1−10 h) at optimum conditions followed by termination of the reaction by heating the reaction mixture and addition of ethanol (3 vol). After centrifugation (10 000 rpm, 15 min), the PHGG sediment was washed thoroughly with ethanol and freeze-dried. The supernatant was concentrated to dryness.

Characterization of PHGG. The molecular mass (M_w) of PHGG was studied by viscometric and GPC methods.

Viscometric Method. The viscosity of PHGG, dissolved in water (0.5%) was measured using an Ostwald viscometer. The average molecular mass (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 $\alpha = 0.747$ (*20*).

GPC. The PHGG solution (1 mL, 0.5%) was loaded onto precalibrated (with T-series Dextrans of defined M_w) Sepharose CL-4B and eluted with water at a flow rate of 18 mL h^{-1}. Each fraction was analyzed for total sugar by the phenol−sulfuric acid method (*21*).

Gas Liquid Chromatography. The galactose/mannose (G/M) content of PHGG was evaluated, after acid hydrolysis followed by derivatization into alditol acetate, by GC on an OV-225 (3% on Chromosorb W) column connected to Shimadzu 8A equipped with a flame ionization detector at 200 °C (*22*).

Viscosity Measurement. The viscosity of PHGG (1% aqueous solution) was measured using a Brookefield viscometer model RV 11 at 20 rpm with spindle number 5 at room temperature.

Infrared Spectroscopy. IR spectral studies were performed using a Perkin-Elmer 2000 spectrometer under dry air at room temperature. PHGG (3 mg) was mixed thoroughly with KBr (200 mg), of which 40 mg was pelletized for taking spectra between 4000 and 200 cm^{-1}.

Solid-State CP-MASS ^{13}C NMR. ^{13}C NMR spectra of GG were recorded on a Bruker dsx$_{300}$ spectrometer (Karlsruhe, Germany) using a cross-polarization pulse sequence. The dry powder (300 mg) placed in a ceramic rotor was spun at the magic angle of 5−7.5 kHz, with the accumulation of more than 2000 scans at a constant time of 2 ms and a pulse (repetition) time of 5 ms.

Characterization of Monomeric Products. The concentrated supernatant from the enzyme digest was studied by HPLC on a precalibrated (using galactose and mannose, 10 μL) μ-Bondapack aminopropyl column (4.1 × 300 mm) using an acetonitrile/water (75:25, v/v) mixture as mobile phase at a flow rate of 1 mL min^{-1} and RI detector.

Texture Profile Analysis. *Preparation of Gel.* Galactomannan/κ-carrageenan mixed gels (1%, total biopolymer) were prepared in a ratio of 1:1 (w/w) by dispersing in deionized water under constant stirring for about 30 min. The mixture was heated at 60 °C for 15 min and poured into a jar of 45 mm diameter and 35 mm height and allowed to set at room temperature for 24 h.

Gel Texture Measurements. Textural parameters, viz. hardness and cohesiveness of mixed gels, were measured by an Instron Texture analyzer equipped with a load cell of 50 N, 35 mm probe, and speed of 20 mm min^{-1} followed by double compression (50%).

RESULTS AND DISCUSSION

The average M_w of native GG, as reported by the manufacturer, was ∼250 kDa, which was in concordance with that derived from GPC and viscometric measurements. Its G/M content was 1:2, and solution viscosity ∼ 4800 cps.

Enzyme Activity. Screening of various carbohydrases, such as pullulanase (*B. acidopullulyticus*), cellulase, pectinase (*Rhizopus* spp.), and amyloglycosidase, for nonspecific action on GG revealed appreciable debranching activity (2.2 units) by pullulanase.

Enzyme Purity. The commercial enzyme preparations usually are admixed with inorganic substances, used as enzyme stabilizers, and they also contain other contaminating proteinaceous matter and, therefore, need prior purification before undertaking further detailed structural studies. Accordingly, the native PAGE of pullulanase showed a major band with some minor bands as contaminants (lane 1 in **Figure 1**B). To rule out the possible contribution of the latter for the nonspecific action, the crude enzyme was subjected to purification by GPC and the fraction (lane 2 in **Figure 1**B), which showed catalytic activity toward both pullulan and GG, was pooled. Native PAGE of the latter corresponded to the earlier major band of crude pullulanase, thus ruling out the involvement of contaminants in its nonspecificity. The purified protein on SDS−PAGE also showed a single band (lane 3 in **Figure 1**B), indicating the protein to be homogeneous and a monomer with a M_w of ∼115 kDa (determined in comparison to standard protein markers; lane 4 in **Figure 1**B). The appearance of a single peak in both HPLC and capillary electrophoresis justified the homogeneity of the purified enzyme (**Figure 2**).

Kinetic Study. The effect of the enzyme concentration (**Figure 3**A) was directly proportional to the increased rate of catalysis of GG and showed a saturation at an enzyme/substrate ratio of 1:70 (w/w; see **Figure 3**A). The nonspecific activity of pullulanase obeyed Michaelis−Menten kinetics (see **Table 1** and **Figure 3**B), with pH optima of 4.5 and temperature optima of 45 °C on GG, whereas for pullulan, it showed pH and temperature optima of 5.5 and 50 °C, respectively. The difference in the pH optima toward specific and nonspecific substrates could be attributed to its pH-dependent conformational changes, as shown by a few proteolytic enzymes on the degradation of chitosan (*23*). Also, the effect of varying the substrate concentration on initial velocity by pullulanase obeyed Michaelis−Menten kinetics. The K_m and V_{max} values obtained from double-reciprocal plot were 6.0 mg mL^{-1} and 1820 nmol min^{-1} mg^{-1}, respectively (**Figure 4**). The substrate concentration above 10 mg mL^{-1} was limited because of its increased viscosity, which hinders easy penetration of the enzyme and therefore restricts enzyme activity. From parts A and B of **Figure 5**, it was evident that the enzyme was stable up to 5 h by retaining ∼90−95% activity, beyond which there was a decline in the activity, indicating maximum stability of the enzyme at pH 4.5 and 45 °C.

PHGG−M_w and Viscosity. Maximum debranching (as seen by decrease in M_w; **Table 2**) of GG occurred within 5 h of incubation with pullulanase. The change in the G/M content agreed well with the nonspecificity of pullulanase toward GG (**Table 2**). A gradual decrease in the viscosity of GG upon co-

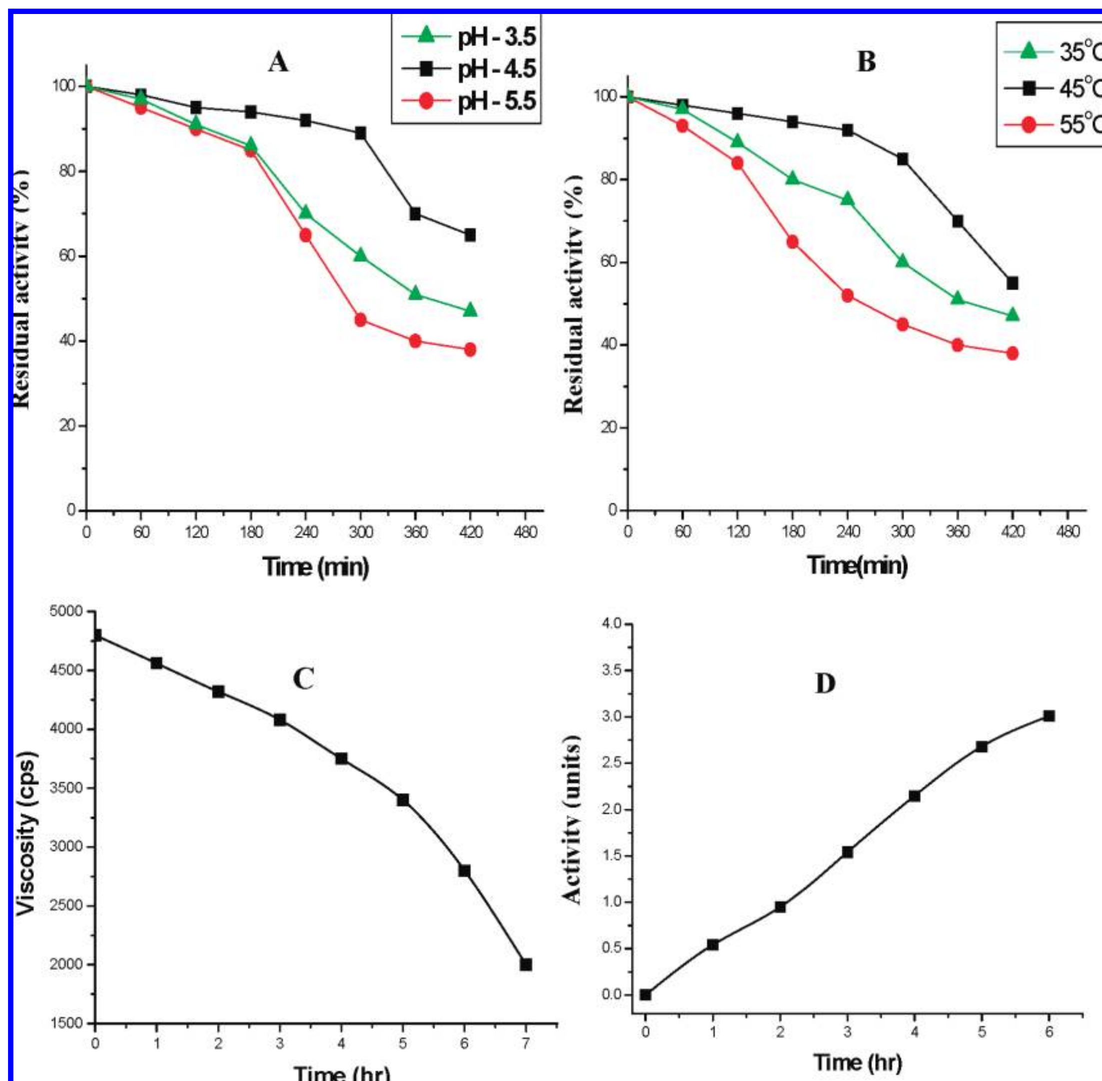

Figure 5. Kinetic parameters: (A) pH stability, (B) temperature stability, (C) GG solution viscosity on co-incubation with pullulanase, and (D) reducing equivalents released during the course of the reaction.

Table 2. Debranching of GG by Pullulanase

	incubation time (h)	molecular weight (kDa)	viscosity (cps)	G/M ratio
native GG	0	240	4800	1:2.0
	1	223	4560	1:2.4
modified GG	3	184	4080	1:3.4
	5	152	3500	1:3.8
	7	110	2600	1:5.1

incubation with the enzyme was also consistent with the increase in reducing equivalents released (parts C and D of Figure **5**).

FTIR Spectroscopy. FTIR analysis of native GG showed a band in the 1200−900 cm^{-1} region because of highly coupled C−C−O stretching of the polymer backbone (*24*). The band at about 1150 cm^{-1} contributing to the vibrations of C−O−C bonds of glycosidic bridges and that at 1077 cm^{-1} because of complex vibrations involving the stretching of the C6−O−C1 bonds, linking the galactose residue to the main chain, were also evident in the spectrum (*25*), and the two bands at 871 and 813 cm^{-1} were characteristic of guar gum (*26*). A gradual decrease in the latter following pullulanase treatment suggests preferential debranching of GG. The change in intensity of the $-CH_2$ (~2930 cm^{-1}) stretching region with sharpening of the absorption band around 1648 cm^{-1} indicated a decrease in molecular size, leading to improvement in solubility of the modified GG.

CP-MASS ^{13}C NMR. Debranching activity of pullulanase on GG was further ascertained by CP-MASS ^{13}C NMR spectroscopy. **Figure 6**A represents the spectrum of native GG with the anomeric signals around 103.810 ppm (C1-Man) and 101.589 ppm (C1-Gal) (*27*). The strong signals dominating around 63−83 ppm correspond to the ring carbon atoms of mannose and galactose. LBG also showed a similar spectrum (**Figure 6**B) with a major difference (upfield shift) in the intensity of the mannosyl C1 signal around 102.3 ppm and the galactosyl C1 signal at 96.5 ppm (*28*). The difference in the intensity was attributed to changes in the G/M ratio. The broadening of the peak observed for the mannosyl C1 signal was due to the sensitivity of the carbon of nonreducing mannose units (*29*). Evidently, the spectrum of modified GG (**Figure 6**C) also revealed considerable changes (upfield shift) in the intensity of C1 regions of Man (102.3 ppm) and Gal (96.85 ppm) because of changes in the G/M ratio, and it was very similar to that of LBG.

GLC as well as HPLC characterization of the supernatant revealed only the presence of galactose, thus strongly supporting the debranching action of pullulanase. During the early hours of the reaction, although the release of galactose was observed,

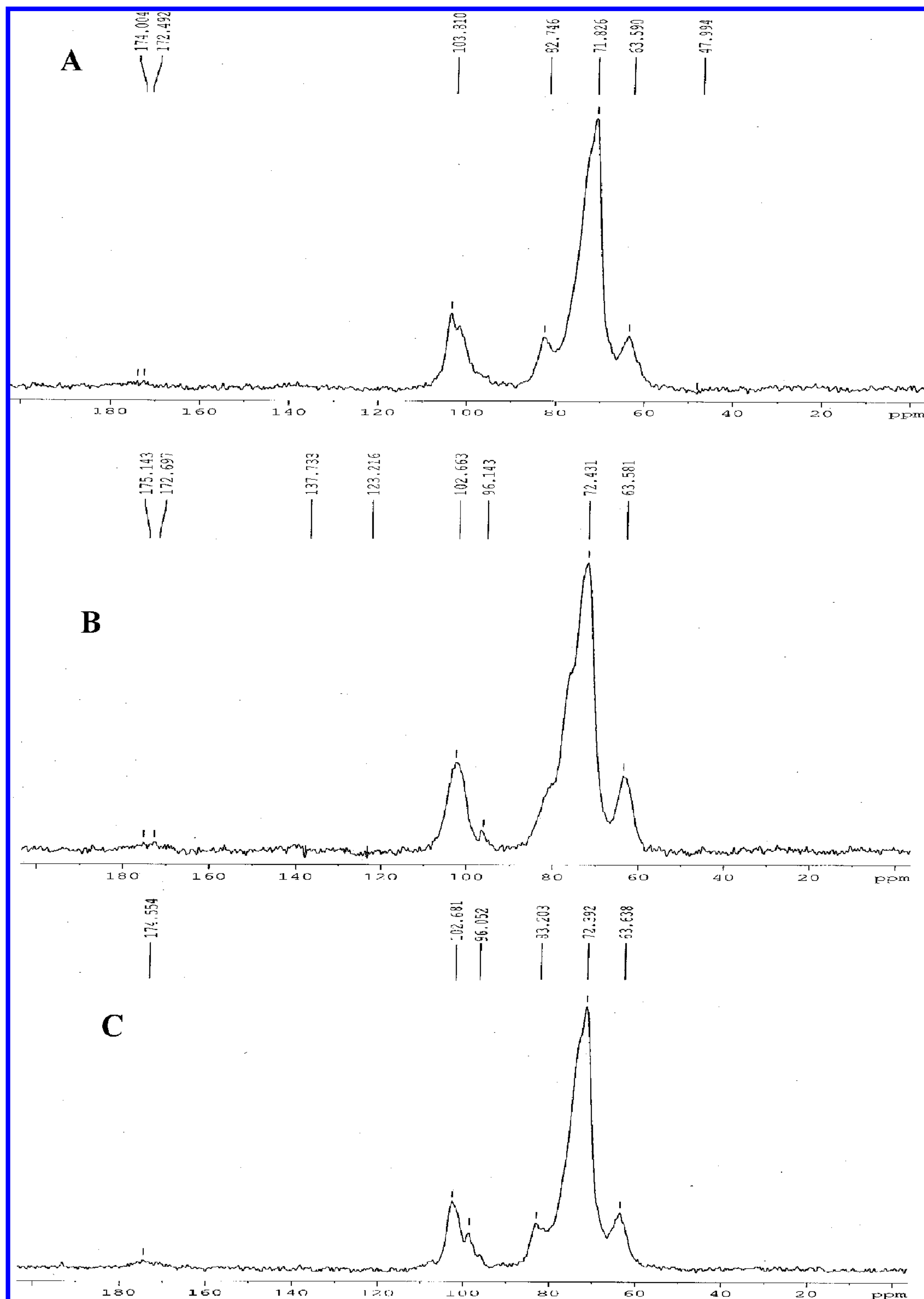

Figure 6. CP-MASS ^{13}C NMR spectra of (A) native GG, (B) LBG, and (C) modified GG.

Table 3. Texture Profile Analysis of Galactomannans—κ-Carrageenan Gels

	hardness (N)	cohesiveness	springeness (mm)	chewiness (N mm)	adhesiveness (N)	gumminess (N)
LBG—κ-carrageenan	10.5	0.067	9.42	6.672	0.043	3.39
GG—κ-carrageenan	NG[a]	NG	NG	NG	NG	NG
modified GG—κ-carrageenan	9.1	0.054	9.10	5.94	0.063	3.68

[a] NG = no gelling.

maximum debranching resulting in modified GG, having a G/M ratio similar to that of LBG, occurring at 5 h of incubation. Subsequently, although the debranching process continued, precipitation of the solution was observed, because of the complete removal of galactose residues, which resulted in an insoluble β-mannan-type core polymer.

Functional Properties of Modified GG. κ-Carrageenan, a less sulfated linear algal polysaccharide made of alternating β-1,3- and α-1,4-linked galactose residues, forms thermoreversible gels, which are brittle in nature. It displays an unique property of synergistic interaction with galactomannans, mainly with LBG forming strong gels, which are of functional utility. Although LBG and GG share common structural similarity of 1,4-linked β-D-mannopyranosyl residues as the backbone attached with α-1,6-D-galactopyranose as the single-residue side chain, the former differs in the G/M ratio of 1:4, respectively. The fine structural analysis of LBG reveals a block-type arrangement of galactosyl residues on the mannan backbone, forming a smooth sequence of the unsubstituted mannose region favoring gelling interaction with other polysaccharides (*30*). In contrast, the uniform arrangement in GG of galactose residues on every second mannose residue results in a highly substituted mannan region, thereby hindering the synergistic interaction with other hydrocolloids.

The native GG on mixing with κ-carrageenan did not show any significant gelling, whereas the pullulanase-treated GG with depleted galactose residues showed a good synergistic interaction upon mixing in a ratio of 1:1 (w/w) with a total biopolymer concentration of 1%. Such mixed gels find applications as a drug delivery system, immobilization of biocatalyst, and gelling and thicking agents. The enzyme-modified GG with reduced galactose content exhibited a strong gelling interaction upon mixing with κ-carrageenan with texture parameters (see **Table 3**) akin to LBG/κ-carrageenan blends. The former could be attributed to the intermolecular interaction of the galactose-depleted region of modified GG with double helices of carrageenan forming a three-dimensional network (*31*−*33*). Texture profile studies of α-galactosidase-treated GG with a decrease in galactose content and showing a strong gelation with κ-carrageenan have been reported (*34*).

To conclude, although there is a possible usage of α-galactosidase in the production of modified GG, its application is limited because of high cost-effectiveness and long operational procedures. The present observation of nonspecific activity of pullulanase from *B. acidopullulyticus* on GG showing preferential debranching provides an alternative route for the production of modified GG. The latter mimics the rheological properties of LBG, which is of considerable commercial utility. Pullulanase is relatively inexpensive and easily available, and tailor-made GG with enhanced functional characteristics can be obtained by controlling the reaction conditions, to meet the needs for various food and nonfood applications.

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