

Isolation and Characterization of an Exopolysaccharide Depolymerase from *Pseudomonas marginalis* HT041B

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Abstract. An enzyme has been isolated from *Pseudomonas marginalis* (a pathogen responsible for the spoilage of fruits and vegetables in storage) that degrades the exopolysaccharide ($m_w > 2 \times 10^6$ Da) produced by this organism. The mechanism of degradation has been determined to be a depolymerization that results from glucosidic cleavage. The product of depolymerization is a polysaccharide of ca. 2.5×10^5 Da. The enzyme has a molecular weight of approximately 28 kDa, a pH optimum of 6.5, $pI = 9.1$, and an apparent K_m of $1.95 \times 10^{-7} \pm 3.5 \times 10^{-8}$ M. Native and enzyme-treated marginalan supported no or very limited growth of the bacterium, respectively.

The structure of the exopolysaccharide (EPS) produced by *Pseudomonas marginalis*, an organism that causes spoilage of stored fruits and vegetables, was recently characterized in our laboratory [10]. This EPS, to which we have given the trivial name marginalan, was shown to contain alternating 1,3-linked β -glucose and α -galactose units (Fig. 1); the galactose is substituted in the 4 and 6 positions with pyruvic acid, and the glucose is substituted with succinic acid at an undetermined position. For the purpose of determining the location of the succinate substituent on glucose by NMR spectroscopy, it was necessary to reduce the viscosity of marginalan solutions. Reducing viscosity by partial depolymerization of the polysaccharide had to be done by a method that would not also result in removal of the succinate. Since we were not able to achieve this by available chemical methods, enzymatic degradation was investigated. Commercially available enzymes such as laminarinase, a β -1,3 glucan hydrolase, had no effect on solution viscosity. Recently, Dunn and Karr [3] reported the presence of a depolymerase in *Bradyrhizobium japonicum* that degrades the *B. japonicum* EPS. Although unusual, other examples of EPS-degrading enzymes isolated from the bacterial species that produce the EPS have been reported [9]. The possibility that *P. marginalis* might contain a depolymerase that would degrade its own EPS

was explored by examining the effect of cell-free extracts and culture supernatant fluids of *P. marginalis* on solutions of marginalan. We found that culture supernatants were inactive; however, the viscosity of marginalan solutions was greatly decreased by the cell-free extracts. The isolation and purification of the *P. marginalis* depolymerase, which we will subsequently refer to as marginalanase, and its properties are reported herein.

Materials and Methods

Protein was measured by the method of Bradford, and carbohydrate by the phenol-sulfuric acid method as previously described [12]. Mass spectra were obtained on a Hewlett-Packard (Avondale, Pennsylvania) Model 5995B gas chromatograph/mass spectrometer (GC/MS) fitted with a Hewlett-Packard 12 m HP-1 capillary column. A Hewlett-Packard Model 1080 instrument was used for HPLC analysis. Preparative isoelectric focusing was carried out on an LKB Model 2117 Multiphor II Electrophoresis unit. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) electrophoresis were carried out on a Phast Separation system (Pharmacia, Piscataway, New Jersey). All dialysis, chromatography, and preparative electrophoresis were done at or below 10°C. Marginalan was prepared as previously described [10].

Enzyme assay. The activity of enzyme fractions was routinely determined by measuring the change in viscosity of a solution of the EPS in an Uberholde viscometer at 30°C. Typically, between 1 and 100 μ l of the enzyme solution was added to 1 ml of a 0.5 mg

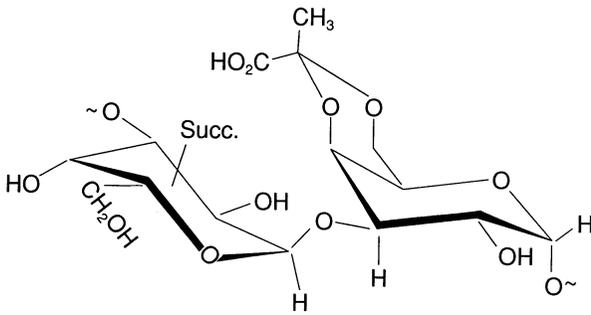


Fig. 1. *Pseudomonas marginalis* exopolysaccharide (marginalan) [10] repeating unit.

ml⁻¹ solution of EPS in 20 mM HEPES buffer, pH 7.4. The solutions were filtered through a 0.45 μ m filter directly into the viscometer. Kinetic data were determined either from initial rates of viscosity change ($-\partial\eta/\partial t$) with respect to time or from normalized half-lives ($\tau_{1/2}$) of the viscous marginalan solutions, calculated from an exponential decay curve-fit by use of a modified Gauss-Newton spreadsheet developed in this laboratory (unpublished data).

Enzyme isolation and purification. *Pseudomonas marginalis* strain HT041B was maintained on *Pseudomonas* agar F at 4°C. For enzyme isolations the bacterium was initially grown at 20°C for 4 days on a modified Vogel and Bonner agar medium [2] with 50.1 g L⁻¹ of gluconate as the carbon source. The resultant growth was scraped from the agar surface and used to inoculate six 2800-ml Fernbach flasks (the content of eleven 100 × 15 mm plates per flask) each containing 1 L of a semi-defined liquid medium consisting of Difco tryptone and yeast extract as well as various salts [4]. Cultures were incubated for 3 days at room temperature with shaking (250 rpm). The cells were then harvested by centrifugation (10,000 g for 30 min at 4°C), washed with distilled water (400 ml), and re-centrifuged. The cells were stored at -20°C until used.

For the preparation of cellular extracts, the frozen cells were suspended in prechilled 50 mM HEPES buffer, pH 7.4. The cells were then broken open by ballistic disintegration with a Bead-Beater apparatus and 0.1-mm diameter glass beads (Biospec Products, Bartlesville, Oklahoma). The beads were allowed to settle, and the supernatant was removed with a pipette. The supernatant fluid was centrifuged at 5,000 g for 5 min at 4°C to remove any remaining glass beads and then at 19,000 g for 45 min at 4°C to remove the remaining cellular debris. The supernatant fluid was fractionally precipitated with ammonium sulfate. Precipitates were collected by centrifugation at 10,000 g (20 min, 10°C) from ammonium sulfate solutions of 25%, 50%, and 75% saturation. The precipitates were dissolved in chilled distilled water, dialyzed against distilled water in 12,000–14,000 molecular weight cutoff tubing, and then lyophilized.

The precipitate obtained after 75% saturation with ammonium sulfate was dissolved in 12.1 mM citric acid–25.7 mM sodium phosphate buffer, pH 5.0, and chromatographed on a CM-Sephacryl ion exchange column with a linear gradient of 0 to 1 M NaCl with the aforementioned buffer as eluant. The active fractions were combined, dialyzed, and lyophilized as described above. This sample was then dissolved in 10 ml of distilled water and

subjected to preparative isoelectric focusing with Pharmalyte pH 8–10.5 ampholytes (Pharmacia) and Sephadex IEF (Sigma, St. Louis, Missouri) at 10°C. The gel was divided into 19 fractions, which were eluted with distilled water. Fractions were dialyzed against 1 M NaCl and then distilled water, followed by concentration on an Amicon (Beverly, Massachusetts) filter (10,000 molecular weight cutoff).

Analytical isoelectric focusing. Precast Phast IEF pH 3–9 gels (Pharmacia) were washed twice (5 min each time) with deionized water followed by deionized (AG501-X8 resin, BioRad, Richmond, California) 10% glycerol for 10 min. After drying to half the original weight (ca. 0.75 g), the gels were rehydrated for 30 min on a rotary shaker with a solution containing 1.53 ml Pharmalyte solution, pH 8–10.5, diluted with 8.64 ml of deionized water.

Molecular weight determination of active protein. Apparent molecular weights were approximated by two methods: (a) gel-permeation chromatography on a Bio-Gel (BioRad) P-200 (100–200 mesh) column (1.5 × 70 cm) with 20 mM HEPES buffer, pH 7.2 as the eluant, and (b) gradient electrophoresis on a PhastGradient 8-25 SDS-PAGE gel (Pharmacia). Both systems were calibrated with molecular weight standards.

Enzyme/substrate products. Substrate, before enzyme treatment, was chromatographed on a Sepharose 2B (Sigma) column (1.5 × 70 cm) to determine the apparent molecular weight. The column was eluted with 20 mM HEPES buffer, pH 7.4, and fractions (3 ml) were monitored for carbohydrate. The enzyme-treated substrate was chromatographed on a Sephacryl S-300 (Pharmacia) column (1.5 × 70 cm) with the same eluant. Both columns were calibrated with appropriate molecular weight standards. The glycosidic bond cleaved by the enzyme was determined by GC/MS analysis of the sodium borodeuteride-reduced, then hydrolyzed, enzyme/substrate products [11].

Enzyme activity staining. The method described by Liao [7] was used to determine the active band on polyacrylamide gels. Agarose gels were prepared by pouring a mixture of 0.8% agarose and marginalan (2 mg ml⁻¹) in 20 mM Tris buffer, pH 7.2, to a thickness of ca. 1 mm in Petri dishes. Immediately after electrophoresis, the polyacrylamide gel was placed, face down, on the agarose gel and tamped gently to remove air bubbles. The plates were covered and incubated overnight at 28°C. The polyacrylamide gel was removed from the agarose, and the agarose was then washed with 1% aqueous mixed alkyltrimethyl ammonium bromide for 15 min. Enzymatic activity was indicated by clear zones against a cloudy white background.

Ability to use marginalan as a sole source of carbon and energy. Starter cultures of strain HT041B were grown overnight in nutrient broth–yeast extract medium (8 g L⁻¹ Difco nutrient broth plus 2 g L⁻¹ of yeast extract, Difco Laboratories, Detroit, Michigan) at 24°C with shaking (250 rpm). Cells were harvested by centrifugation, washed once with sterile distilled water, and resuspended in sterile water to give an optical density at 600 nm (OD_{600nm}) of 1.0. This suspension (0.1 ml per tube) was used to inoculate *Pseudomonas* minimal medium [13] (3 ml per sterile 1 × 9.5-cm culture tube) containing the following carbon sources: glucose (0.1%), marginalan (0.1%), or marginalanase-treated marginalan (0.1%). The marginalanase-treated marginalan had a viscosity equal to water when viscosity was tested as

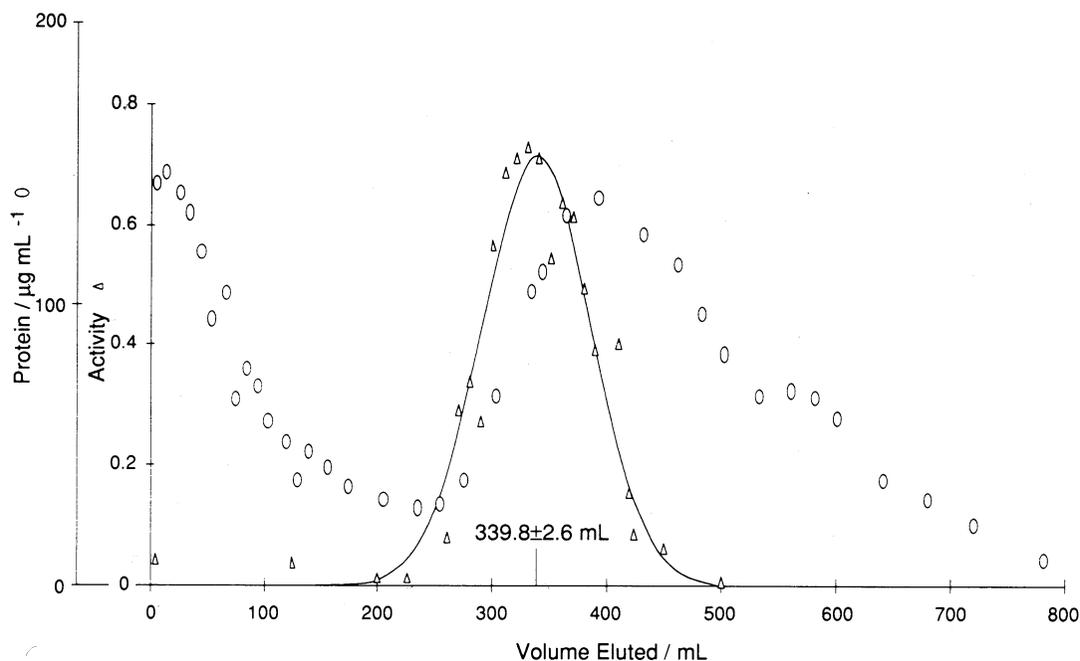


Fig. 2. CM-Sepharose chromatography of marginalanase. Relative activity (A) data fit well to a Gaussian line shape where $A = A_{\max} \text{EXP}[-\{(V_o - V_i)^2 / (2 s^2)\}]$; in this relationship $A_{\max} = 0.72 \pm 0.03$, V_o (peak center) = 340 ± 3 ml and s (standard deviation) = 48 ± 3 ml; o = protein, Δ = relative activity.

described above. Cultures were incubated at 28°C with shaking (250 rpm), and bacterial growth was followed by periodically determining $\text{OD}_{600\text{nm}}$.

Removal of substrate substituents. Succinate was removed by treatment with base, and pyruvate by treatment with oxalic acid as previously described [10].

Results

A summary of the purification of marginalanase is given in Table 1, and results of the CM-Sepharose chromatography are shown in Fig. 2. The active enzyme fraction from preparative isoelectric focusing gave a single band on electrophoresis (Fig. 3a, lane 3); small amounts of ampholytes that were not removed by dialysis were also observed. Activity staining (Fig. 3b) confirms that this band is a depolymerase.

The molecular weight of the enzyme, as determined by SDS-PAGE electrophoresis and gel filtration, is ca. 28,000 Da, and its isoelectric point, as determined by isoelectric focusing, is 9.1. The pH optimum for depolymerase activity was determined to be between pH 6.5 and 7.5. The enzyme is inactive below pH 4 and above pH 8; it is unstable above 40°C. Two methods of analysis, based on viscosity change, gave similar K_m s (0.39 ± 0.07 mg

Table 1. Purification of marginalanase

Fraction	Protein (mg)	Specific activity ^a
Cell-free extract	742	2.97×10^2
(NH_4) ₂ SO ₄ ppt (75%)	310	5.07×10^2
CM-Sepharose active fraction	11	9.20×10^3
IEF active fraction	0.084	2.07×10^5

^a A unit of activity is defined as: $\{\tau_0 - \tau_{15}\} / \tau_0 = 0.2$, where τ_0 and τ_{15} are the Uberholde viscometer drainage times without enzyme and 15 min after the addition of enzyme to the substrate solution, respectively.

ml^{-1} , $\sim 1.95 \times 10^{-7} \pm 3.5 \times 10^{-8}$ M; Fig. 4). The enzyme did not hydrolyze laminarin, citrus pectin, xanthan gum, guar gum, locust bean gum, or bacterial alginate. It maintained activity against succinate-depleted marginalan. Removal of the pyruvate substituent from the substrate resulted in EPS solutions whose viscosity was too low, even at concentrations as high as 3 mg ml^{-1} , to determine activity for this substrate.

The products of depolymerization were examined by gel filtration chromatography and GC/MS analysis (Fig. 5). The untreated EPS eluted in the void volume of the Sepharose 2B column, suggest-

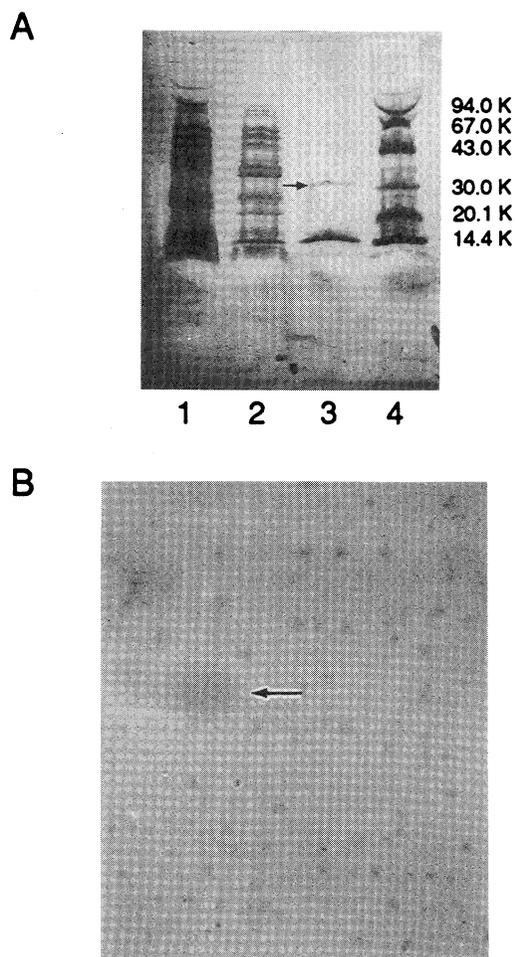


Fig. 3. (A) SDS electrophoretic analysis of enzyme fractions. Lane 1, $(\text{NH}_4)_2\text{SO}_4$ ppt; lane 2, after cation exchange; lane 3, after preparative IEF; lane 4, molecular weight standards. (B) Activity-stained gel.

ing a molecular weight greater than 2×10^6 . After 48 h of incubation (additional enzyme was added after 24 h), the enzyme-treated EPS had an apparent average molecular weight of ca. 2.5×10^5 as determined by chromatography on a Sephacryl S-300 column.

After enzyme treatment, the reducing end of the resultant polysaccharide was determined by GC/MS end-group analysis [11] to be glucose (Fig. 5). The results of this analysis indicate that the molecular weight of the partially degraded polysaccharide was ca. 3×10^5 .

Pseudomonas marginalis strain HT041B was not capable of utilizing native marginalan as a sole source of carbon. With glucose as the carbon source, the $\text{OD}_{600\text{nm}}$ readings of the cultures after 21

and 48 h of incubation averaged 0.700 and 0.606, respectively. In contrast to native marginalan, the $\text{OD}_{600\text{nm}}$ readings averaged 0.035 and 0.049 after 21 and 48 h, respectively. Depolymerase-treated marginalan was able to support only very limited growth with average $\text{OD}_{600\text{nm}}$ readings of 0.173 and 0.121 at 21 and 48 h, respectively. Addition of marginalase alone to culture media in an equivalent amount as that used to treat the native marginalan did not support any bacterial growth.

Discussion

Except in rare instances [1], the product of enzymatic glycolytic cleavage of bacterial EPS is the oligosaccharide repeating unit. To our knowledge, there is only one example in the literature where a homogeneous EPS is not degraded to repeating unit oligomers. Dunn and Karr [3] recently described the isolation of a depolymerase of *Bradyrhizobium japonicum* EPS that degrades this polysaccharide to relatively high-molecular-weight fragments rather than the repeating unit, as determined by reducing end-group measurements.

That marginalanase actually lowered solution viscosity of marginalan by depolymerization could not be confirmed by chemical end-group analysis [6] because of the small quantity of end groups released upon enzymatic treatment of the substrate; the molecular weight of the EPS was reduced from $>2 \times 10^6$ to only 2.5×10^5 Da by treatment with the enzyme. Therefore, 1 mg of substrate (5×10^{-10} moles of reducing sugar) would yield only 4×10^{-9} moles of reducing sugar and provides an insufficient end-group concentration for reliable analysis. Solubility and viscosity limitations precluded higher substrate concentrations. A GC/MS method was developed to obtain direct evidence that glycolytic cleavage was the mechanism of enzyme action [11]. The results from this analysis confirmed that marginalan is a glycolytic enzyme, specifically an endoglyconase.

Except for its high isoelectric point, marginalanase has properties similar to those of other glyconases [5]. The high isoelectric point, which was an obstacle in obtaining pure enzyme, was overcome by using high pH ampholyte solutions (pH 8–11.5) in the final preparative isoelectric focusing step. The yield of pure enzyme was low enough to require the application of significant portions (1–2%) of the fractions obtained by preparative isoelectric focusing to observe protein bands by electrophoretic analysis even with silver staining tech-

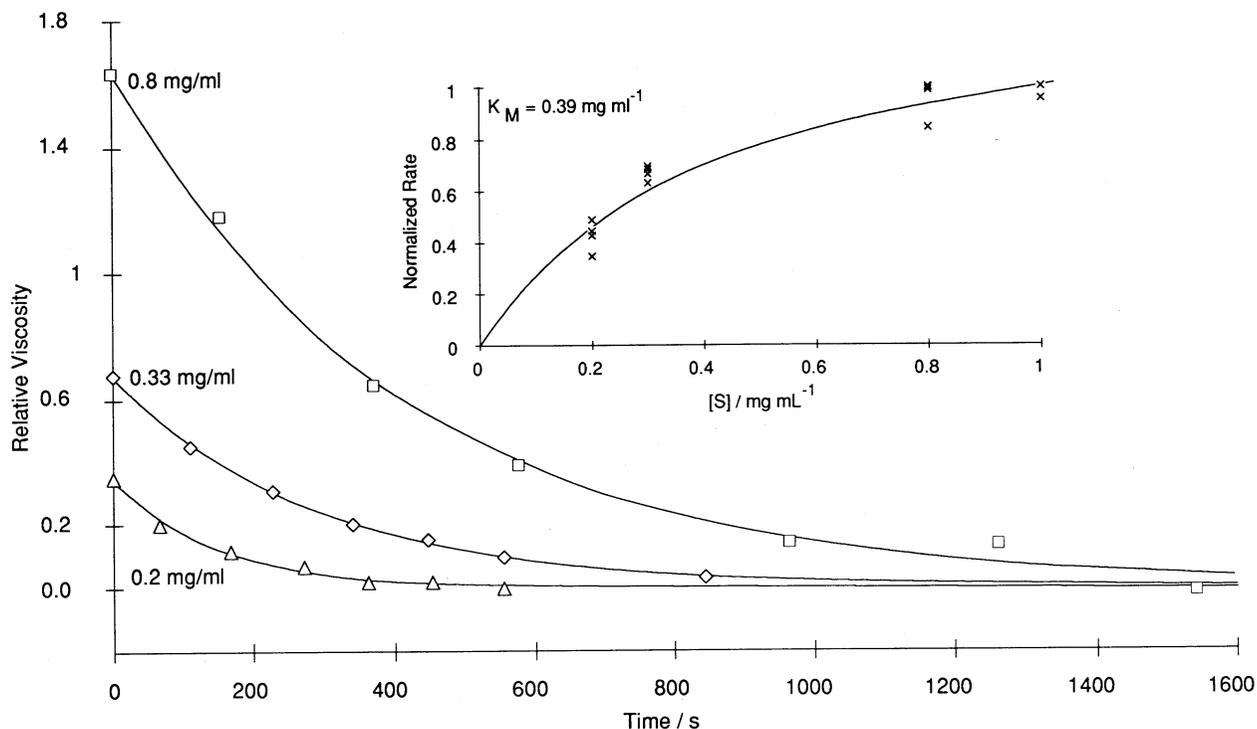


Fig. 4. Exponential fits for changes in relative viscosity with respect to time for 0.8, 0.33, and 0.2 mg ml⁻¹ marginalanal at 30°C. Insert figure: best fit of the experimental rate data to the Michaelis-Menton equation; complete experiments were replicated twice.

niques. Activity, on the other hand, could be located with as little as 0.03% of the fractions owing to the high specific activity of the enzyme.

The approximate value for the K_m , 0.39 mg ml⁻¹ (ca. 1.95×10^{-7} M), which was determined from both initial $-\partial\eta/\partial t$ and normalized $\tau_{1/2}$ for various concentrations of marginalanal, appears reasonable for a substrate with such a high molecular weight ($>2 \times 10^6$ Da). It was demonstrated (results not shown) that the products of the substrate-enzyme interaction did not contribute to solution viscosity, but were directly related to substrate concentration, validating the use of this technique to measure relative enzyme activity. Further characterization of the enzyme will have to be delayed until we are able to isolate larger quantities of the pure enzyme. Although glycolytic cleavage occurs at the glucose-to-galactose bond, it is not clear why the reaction does not proceed to the disaccharide repeating unit, or some low multiple of the repeating unit. It is possible that the EPS's enzyme-binding site contains a modified structure in the repeating unit, e.g., a depyruvylated galactose, that would not be apparent in the structural analysis. A more

detailed analysis of the reaction products and enzyme-substrate complex is necessary to determine the mechanism of activity of this unusual enzyme. It would also be interesting to determine whether marginalanase can depolymerize the exopolysaccharides similar to marginalan isolated from *Rhizobium meliloti* strains YE-2 [8] and RM 1021 [6].

One can only speculate on the function of this enzyme in the bacterium. Marginalan does not appear to be a readily utilizable source of carbon and energy for strain HT041B, since native and depolymerized marginalan supported little to no growth of this strain in a completely defined medium. Although marginalanase activity was not observed in culture fluids, the enzyme may be surface bound and function only in the immediate environment of the bacterium. By regulating the molecular weight of the EPS, the enzyme could conceivably be controlling the viscosity of the bacterial environment, which may be an important factor in the viability of the microorganism. The enzyme may also act to release bacterial cells from biofilms for the purpose of colonizing new surfaces when nutrients are no longer limiting.

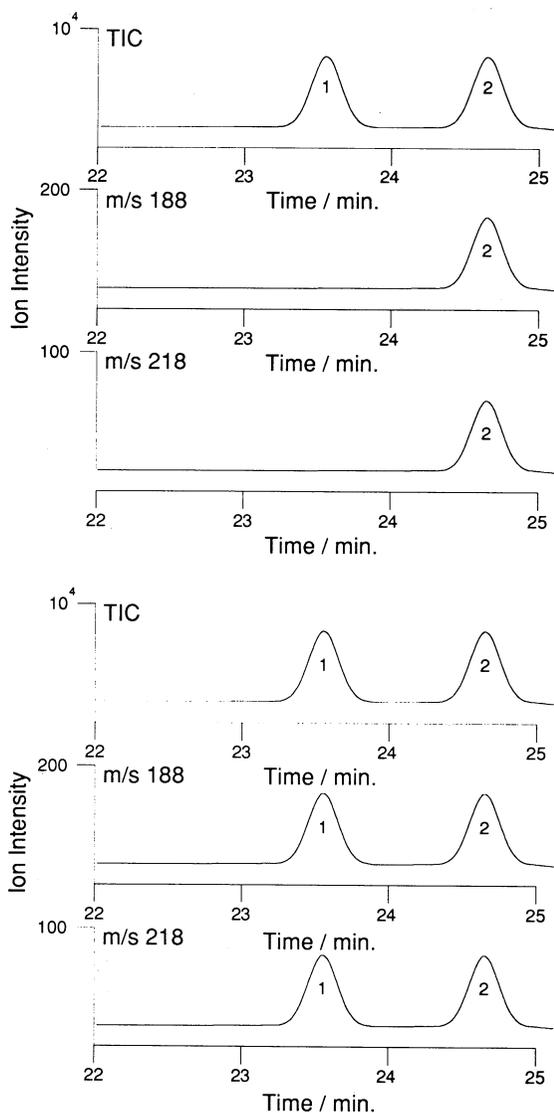


Fig. 5. GC/MS analysis of: (a) marginalanase-treated marginalan; (b) acid-hydrolyzed marginalan. TIC = total ion chromatogram; 1 = galactitol acetate; 2 = glucitol acetate; M/Z = mass to charge ratio.

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