Biotechnology Letters (2006) 28: 241–245
DOI 10.1007/s10529-005-5525-0

Polymeric and compositional properties of novel extracellular microbial polyglucosamine biopolymer from new strain of *Citrobacter* sp. BL-4

Lin-Su Kim, Soo-Jung Hong, Mi-Kyung Son & Yong-Hyun Lee*
Department of Genetic Engineering, College of Natural Sciences, Kyungpook National University, Daegu 702-701, S. Korea
**Author for correspondence (Fax: +82-53-959-8314; E-mail: leeyh@knu.ac.kr)*

Received 20 September 2005; Revisions requested 6 October 2005; Revisions received 16 November 2005; Accepted 16 November 2005

Key words: Citrobacter sp. BL-4, microbial polyglucosamine biopolymer, pH-stat fed batch cultivation, polymer composition, polymeric properties

Abstract

A novel polyglucosamine polymer, PGB-2, was produced extracellularly from a new strain *Citrobacter* sp. BL-4 using pH-stat fed batch cultivation. It was composed of 97.3% glucosamine and 2.7% rhamnose; its average molecular weight, solubility in 2% acetic acid and viscosity were 20 kDa, 5 g l^{-1} and 2.9 cps, respectively. FT-IR and ^{1}H NMR spectra of PGB-2 revealed a close identity with chitosan from crab shells.

Introduction

A variety of microbial exopolysaccharides have been developed. Some, such as curdlan, dextran, gellan, pullulan, and xanthan (Sutherland 1998, 2001), have practical applications. Recently, a unique bioflocculant composed of 29.4% hexosamine with an M_w of 320 kDa was produced extracellularly from *Citrobacter* sp. TKF04 for the treatment of sludge from wastewater (Fujita *et al.* 2000, 2001, Jang *et al.* 2001).

Previously we have described a new extracellular polyglucosamine biopolymer from a new strain of *Enterobacter* sp. BL-2 (Son *et al.* 2005). It was composed of 95% hexosamine with an M_w of 105 kDa, and its structural feature was similar to that of chitosan from natural crab shells. The microbial polyglucosamine biopolymer has some potential usefulness as a substitute of chitosan for the food, pharmaceutical, and agricultural industries (Shahidi *et al.* 1999, Thanou *et al.* 2001).

In this study, another microbial polyglucosamine biopolymer was produced by pH-stat fed batch cultivation using a new strain *Citrobacter* sp. BL-4. The polymeric and compositional properties of a new microbial polyglucosamine biopolymer PGB-2 was determined after three-step purification. The structural similarity of PGB-2 with those of chitosan from crab shells was also compared using FT-IR and ^{1}H NMR.

Materials and methods

Screening of bacteria and phylogenetic analysis

Bacteria forming mucoid and ropy colonies were first selected after cultivation on LB agar medium (pH 7.5) supplemented with 1% (w/v) glucose and 0.5% sodium acetate at 30 °C for 72 h, and then re-cultivated in 10 ml basal liquid medium for 48 h. A strain, BL-4, secreting a polymer with a high-hexosamine-content, was finally selected. The partial 16S rDNA of isolate was cloned by a PCR using the primer sets, BSF-I (5′-TAACACATGCAAGTC-3′) and BSR1407 (5′-GACGGGCGGTGTGTAC-3′), and then a phylogenetic tree was constructed according to the neighbor-joining method using Clustal W version 1.75 [http://www.genebee.msu.su].

pH-stat fed batch cultivation of Citrobacter *sp. BL-4*

Citrobacter sp. BL-4 was cultured in a liquid medium (Jang *et al.* 2005) composed of 1.5% (w/v) sodium acetate, 0.1% $(NH_4)_2SO_4$, 0.01% yeast extract, 2% $CaCl_2$, 0.5% $FeCl_3{\cdot}6H_2O$, and 0.05% trace elements at 30 °C, 500 rpm, and 0.5 vvm for 96 h. A 5 M acetic acid solution was fed to maintain pH 8.0 for overproduction of microbial polyglucosamine biopolymer PGB-2.

Determination of molecular weight and hexosamine content

The molecular weights (M_w, M_n) of PGB-2 were measured by gel permeation chromatography using a breeze HPLC system equipped with an ultrahydrogel column and RI detector (Waters, Milford, USA) with pullulan polysaccharides as standards (Phenomnex Inc., Torrance, USA). The hexosamine content was determined by the modified Elson-Morgan method using glucosamine as the standard after modification (Jang *et al.* 2001, 2005).

Analysis of element and monosaccharide compositions

A microbial polyglucosamine biopolymer PGB-2 was hydrolyzed with 6 M HCl at 105 °C for 10 h, neutralized with the same volume of 6 M NaOH, and then the monosaccharide compositions were determined by an HPLC system (Gilson, Villiers-Le-Bel, France) equipped with an OP-NH2 column (RStech Co., Daejeon, S. Korea) using acetonitrile/water (7:3, v/v) as the mobile phase at 1 ml min^{-1}.

FT-IR and FT-NMR analysis of microbial polyglucosamine biopolymer

The FT-IR spectrum was analyzed by FT-IR spectroscopy (Perkin-Elmer Inc., Norwalk, USA), and the ^1H NMR spectrum was obtained using FT-NMR spectroscopy (Bruker, Rheinstetten, German) at 400 MHz with an acquisition time of 1.3 s, comparing with those of chitosan from crab shells (Sigma) fully deacetylated using 2 M HCl.

Results and discussion

Selection and identification of new strain excreting microbial polyglucosamine biopolymer

A bacterial strain BL-4 excreting a biopolymer with high-hexosamine-content was newly screened from a fermentation broth. The new strain was a straight rod-shaped bacterium, and revealed a high 16S rDNA homology with strains belonging to the genus *Citrobacter* showing the highest homology of 99.3% with *Citrobacter freundii*. A strain BL-4 was classified as *Citrobacter* sp. BL-4, and then deposited as a new strain under KACC91177.

pH-stat fed batch cultivation of new Citrobacter *sp. BL-4 and purification of biopolymer*

Citrobacter sp. BL-4 is a facultative anaerobe that preferably assimilates acetic acid. Thus, it was cultivated using a pH-stat fed-batch method adding 5 M acetic acid occasionally to maintain the medium pH at 8.0 to achieve over-production of the polyglucosamine biopolymer. As shown in Figure 1, 4.86 g of the crude biopolymer l^{-1} was produced extracellularly after 84 h.

Crude biopolymer was purified using the three-step procedure: ethanol precipitation, followed by first and second deproteinization using 2 M NaOH. As shown in Table 1, the recovery yields at each purification step were 92%, 73% and 67%, respectively. The residual protein in

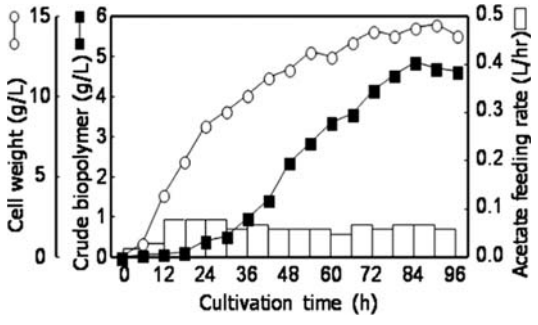

Fig. 1. pH-stat fed batch cultivation of *Citrobacter* sp. BL-4 for excretive production of microbial polyglucosamine biopolymer PGB-2. The strain was cultivated in a basal medium containing 1.5% (w/v) sodium acetate, 0.1% $(NH_4)_2SO_4$, 0.01% yeast extract, 2% $CaCl_2$, 0.5% $FeCl_3{\cdot}6H_2O$, and 0.05% trace elements, at 500 rpm, 0.5 vvm for 96 h. 5 M acetic acid solution was added fed batch wisely to maintain pH 8.0.

Table 1. Purification of microbial polyglucosamine biopolymer PGB-2 excreted from Citrobacter sp. BL-4.

Purification step	DBW[a] (g l^{-1})	Yield (%)	Composition (%)		
			Hexosamine	Protein	Others
Crude biopolymer	4.86	100	75.8	15.4	8.8
Ethanol precipitation	4.48	92	80.2	13.4	6.4
1st NaOH treatment	3.55	73	89.2	5.1	5.7
2nd NaOH treatment	3.28	67	97.3	0.0	2.7

[a]DBW: Dried biopolymer weight.
The excreted PGB-2 was precipitated using four volumes of absolute ethanol at 4 °C for 24 h, and deproteinized twice with 2 M NaOH at 121 °C for 10 min, and neutralized with the same volume of 2 M HCl, and then lyophilized after dialysis against distilled water.

crude biopolymer was successfully removed after two-step NaOH treatment, and the hexosamine content was increased from 75.8% of a crude biopolymer to 97.3% after the three-step purification.

Polymeric properties of microbial polyglucosamine biopolymer PGB-2 excreted from new Citrobacter *sp. BL-4*

The homogeneity of the purified microbial polyglucosamine biopolymer PGB-2 was confirmed by a compact single peak in a gel permeation chromatogram as shown in Figure 2. The weight-average molar mass (M_w) of PGB-2 was measured to be 20 kDa, a much lower value compared to other known microbial polyglucosamine biopolymers, such as the bioflocculant from *Citrobacter* sp. TKF04 with M_w of 320 kDa (Fujita *et al.* 2000) and the polyglucosamine biopolymer PGB-1 excreted from *Enterobacter* sp. BL-2 with M_w of 105 kDa reported in our previous work (Son *et al.* 2005). The number-average molecular mass (M_n) was 16 kDa, and the polydiversity (M_w/M_n) was calculated to be 1.2, implying a near-homogeneous polymer.

The solubilities of microbial polyglucosamine biopolymer PGB-2 in water and 2% acetic acid solution were 1 and 5 g l^{-1}, respectively, and the viscosity of PGB-2 fully dissolved in acetic acid solution was 2.9 cps, a very low value compared to 4.95 cps for microbial polyglucosamine biopolymer PGB-1 from *Enterobacter* sp. BL-2 and 10.8 cps for chitosan from crab shells (data not shown).

Element and monosaccharide compositions of microbial polyglucosamine biopolymer PGB-2

The elemental composition of PGB-2 is given in Table 2. It was composed of only two monosaccharides; 97.3% glucosamine and 2.7% rhamnose, with a molar ratio of 89:1. This indicates that PGB-2 is a near-homogeneous biopolymer consisting predominantly of glucosamine with a small amount of rhamnose, similar with chitosan from

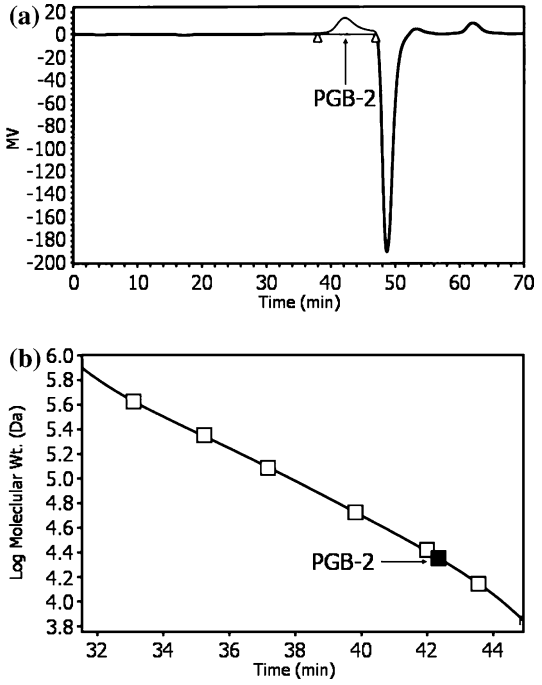

Fig. 2. Gel permeation chromatogram of PGB-2 for M_w measurement (a) and calibration curve of pullulan standards (b). The PGB-2 peak was detected at 42.13 min and others were solvent peaks.

Table 2. Element and monosaccharide compositions of purified microbial polyglucosamine biopolymer PGB-2.

	%, w/w	molar ratio
Element composition		
Carbon	37.2	
Hydrogen	7.2	
Oxygen	48.7	
Nitrogen	6.9	
Monosaccharide composition		
Glucosamine	97.3	88.9
Rhamnose	2.7	1.0

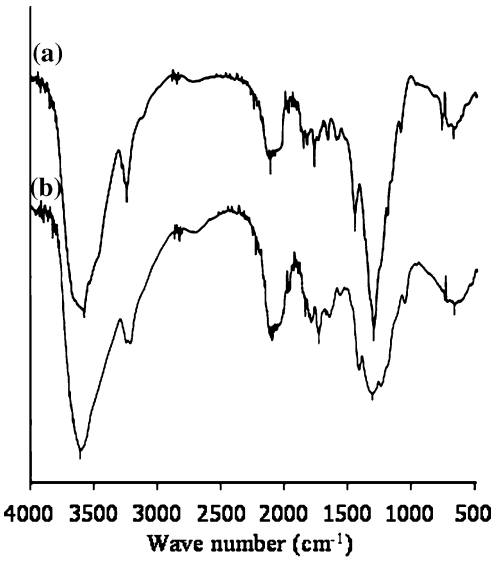

Fig. 3. Comparison of FT-IR spectra for microbial polyglucosamine biopolymer PGB-2 (a) and chitosan from crab shells (b).

crab shells which consists of D-glucosamine with only a small amount of *N*-acetyl- D-glucosamine.

FT-IR and FT-NMR spectra for microbial polyglucosamine biopolymer PGB-2

As shown in Figure 3, the FT-IR spectrum for PGB-2 was composed of OH band at 3395 cm^{-1}, CH band at 2892 cm^{-1}, CH_2 band at 1396 cm^{-1}, CH_3 band at 1169 cm^{-1} and primary amide band at 1642 cm^{-1}, the functional bands in the similar positions of chitosan (Prashanth *et al.* 2002, Velde *et al.* 2004).

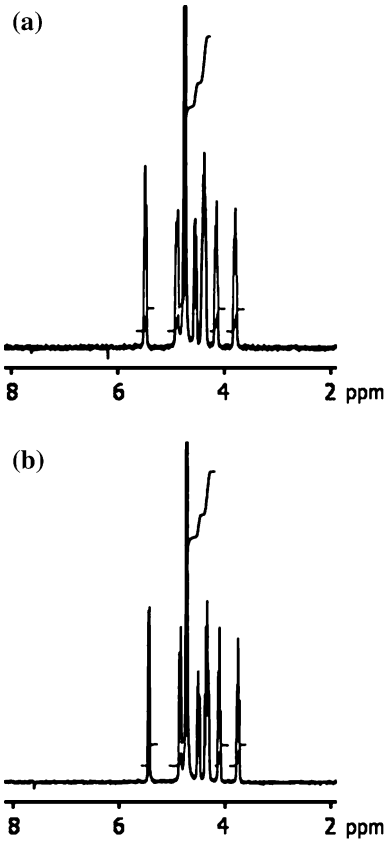

Fig. 4. ^1H NMR spectra for microbial polyglucosamine biopolymer PGB-2 (a) and chitosan from crab shells (b).

In the ^1H NMR spectrum, the main hydrogen peaks appeared at 5.5, 4.89, 4.13–4.75 and 3.8 ppm as shown in Figure 4. The ^1H NMR spectrum for PGB-2 was also mostly identical to that for a fully deacetylated chitosan, even including no appearance of H-Ac peak at 2–2.5 ppm that reflects the proton in the acetylated form of glucosamine (Lavertu *et al.* 2003).

In conclusion, the microbial polyglucosamine biopolymer PGB-2 secreted from a new *Citrobacter* sp. BL-4 had similar structural features to the deacetylated form of chitosan from crab shells. The microbial polyglucosamine biopolymer PGB-2 with low molecular weight and low viscosity seems to be potentially useful as a chitosan substitute in the food, pharmaceutical, and agricultural industries, such as, the drug delivery system and antioxidants.

Acknowledgements

This work was supported by a grant (20050401-034-639-192-01-00) from BioGreen 21 Program, Rural Development Administration, Republic of Korea. Authors are grateful for technical advice of Dr Jin-Ho Jang.

References

Fujita M, Ike M, Tachibana S, Kitada G, Kim MS, Inoue Z (2000) Characterization of a bioflocculant produced by *Citrobacter* sp. TKF04 from acetic and propionic acids. *J. Biosci. Bioeng*. **89**: 40–46.

Fujita M, Jang JH, Kim MS, Hirao T (2001) Bioflocculation production from lower-molecular fatty acids as a novel strategy for utilization of sludge digestion liquor. *Water Sci. Technol*. **44**: 237–243.

Jang JH, Ike M, Kim MS, Fujita M (2001) Production of a novel bioflocculant by fed-batch culture of *Citrobacter* sp. *Biotechnol. Lett*. **23**: 593–597.

Jang JH, Hia HC, Ike M, Inoue C, Fujita M, Yoshida T (2005) Acid hydrolysis and quantitative determination of total hexosamines of an exopolysaccharides produced by *Citrobacter* sp. *Biotechnol. Lett*. **27**: 13–18.

Lavertu M, Xia Z, Serreqi AN, Berrada M, Rodrigues A, Wang D, Buschmann MD, Gupta A (2003) A validated ^{1}H NMR method for the determination of the degree of deacetylation of chitosan. *J. Pharm. Biomed. Anal*. **32**: 1149–1158.

Prashanth KVH, Kittur FS, Tharanathan RN (2002) Solid state structure of chitosan prepared under different N-deacetylating conditions. *Carbohydr. Polym*. **50**: 27–33.

Shahidi F, Arachchi JKV, Jeon YJ (1999) Food applications of chitin and chitosans. *Trends Food Sci. Tech*. **10**: 37–51.

Son MK, Shin HD, Huh TL, Jang JH, Lee YH (2005) Novel cationic microbial polyglucosamine biopolymer from new *Enterobacter* sp. BL-2 and its bioflocculation efficacy. *J. Microbiol. Biotechnol*. **15**: 626–632.

Sutherland IW (1998) Novel and established applications of microbial polysaccharides. *Tibtech*. **16**: 41–46.

Sutherland IW (2001) Microbial polysaccharides from Gram-negative bacteria. *Int. Dairy J*. **11**: 663–674.

Thanou M, Verhoef JC, Junginger HE (2001) Chitosan and its derivatives as intestinal absorption enhancers. *Adv. Drug Deliv. Rev*. **50**: S91–S101.

Velde K, Kiekens P (2004) Structural analysis and degree of substitution of chitin, chitosan and dibutyrylchitin by FT-IR spectroscopy and solid state ^{13}C NMR. *Carbohyd. Polym*. **58**: 409–416.