ELSEVIER

Polymer 43 (2002) 1301–1305

polymer

www.elsevier.com/locate/polymer

Double helix of *Agrobacterium tumefaciens* succinoglycan in dilute solution ☆

Isamu Kaneda*, Atsushi Kobayashi, Kazuyuki Miyazawa, Toshio Yanaki

Basic Research Center, Shiseido Co., Ltd, 2-2-1 Hayabuchi, Tsuzuki-Ku, Yokohama, Japan 224-8558

Received 4 June 2001; received in revised form 6 September 2001; accepted 25 September 2001

Abstract

Succinoglycan samples ranging in weight-average molecular weight from 1.4×10^5 to 10.5×10^5 (in 0.1 M aqueous NaCl at 25°C), prepared from native succinoglycan by sonication, were investigated by static light scattering and viscometory in 0.1 M aqueous NaCl. It is well known that the conformation of the polysaccharide changes at around 65°C. In order to confirm the conformation of the polysaccharide, the property of the polysaccharide in dilute solution was studied at both 25 and 75°C. The molecular weight of a sample at 25°C is the double of the value at 75°C. The molecular weight dependence of $\langle s^2 \rangle_Z$ and $[\eta]$ for succinoglycan shows that the polysaccharide is a rod-like polymer at 25°C and behaves like a semi-flexible polymer at 75°C in 0.1 M aqueous NaCl. Moreover, the linear mass density of the polysaccharide was almost twice that expected for single succinoglycan molecules. These results indicate that the conformation of the polysaccharide would be a double helix at 25°C and the helix melts to a single strand behaving as a semi-flexible chain above 65°C.

Keywords: Succinoglycan; Ordered conformation; Dilute solution

1. Introduction

Succinoglycan is a microbial polysaccharide, such as Xanthan and Schizophyllan. Succinoglycan was isolated from soil as a bacterium named *Alcaligenes faecalis* var. *myxogenes* by Harada [1]. Succinoglycan is also produced by other bacteria, e.g. *Rhizobium* [2,3], *Alcaligenes*, *Agrobacterium*, or *Pseudomonas* [4]. Succinoglycan consists of octasaccharide repeating units [5,6]. The content of the residues varies with the producing bacteria species and/or its cultivation condition. The backbone of the succinoglycan consists of repeating unit of four monosaccharides (three D-glucose and one D-galactose residue), where galactosylated glucose residue serves as a chain composed of D-glucose residues at C-6. Pyruvate residue is esterificated to D-glucose at the end of the side chain. Succinate residue is located on an unknown position in the side chain region. In this study, a succinoglycan produced by *Agrobacterium tumefaciens* was used. Fig. 1 shows the chemical structure of the succinoglycan. The molar ratio of the repeating unit, D-glucose, D-galactose, pyruvate, and succinate residues are $7/1/0.5 \backsim 1/0.5 \backsim 1$, respectively [7].

The polysaccharide is interesting in industrial application due to its high potential as a water-soluble thickener [8]. Viscosity of the polysaccharide solution is very high even in low concentration and can be preserved even in high salt or acidic condition. Therefore, it is a useful thickener for recovering oil [9,10] and produced in large scale by fermentation procedure.

It is reported that the aqueous solution of succinoglycan dramatically reduces its viscosity at around 65°C [11,12]. The phenomenon is thought to be due to order–disorder conformational transition. Since the ordered conformation could become a disordered one above 65°C, the viscosity of the polysaccharide solution is reduced. A rheological study [13] proposes that a succinoglycan has a rigid structure. It is reported that an ordered conformation is speculated to be a single helix by calorimetry [14,15]. Recently, the single helix of the polysaccharide has been observed by atomic force microscopy [16]. On the other hand, Burova et al. [17] put forward the proposal that the thermally induced conformation transition is the dissociation of a double helix followed by the melting of the dissociated single helix. These different proposals may arise from approximations or assumptions underlying the theories used. The most reliable approach to the experimental determination of the

☆ Presented in part at the Annual Meeting of American Chemical Society, Boston, MA, August 1998.

* Corresponding author. Tel: +81-45-590-6053; fax: +81-45-590-6088.
E-mail address: isamu.kaneda@to.shiseido.co.jp (I. Kaneda).

0032-3861/02/$ - see front matter
PII: S0032-3861(01)00652-8

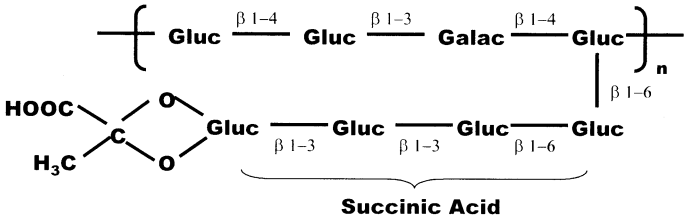

Fig. 1. Chemical structure of succinoglycan.

conformation of a semi-flexible polymer would be to analyze the molecular weight dependence of such properties as the intrinsic viscosity and the mean-square radius of gyration [18]. However, there are insufficient numbers of reports related to the direct comparison between order and disorder conformation of the polysaccharide in dilute solution.

The aim of this work is to clarify the details of order–disorder conformational change of the succinoglycan in dilute solution. If conformational data of the succinoglycan at both temperatures is obtained, the molecular characteristic of the polysaccharide will be clarified. The dilute solution study, static light scattering measurement and viscometry were done at both 25 and 75°C. The conformation was evaluated by the molecular weight dependence of the radius of gyration and to the intrinsic viscosity. Moreover, the linear mass density and the persistence length of the polysaccharide were also estimated by using a hydrodynamic worm-like chain model.

2. Experimental

2.1. Materials

The succinoglycan used in this study was purchased from Rodia Nikka (Tokyo, Japan). In order to make various molecular weights of the polysaccharide, a 0.1 M aqueous NaAc solution of the crude succinoglycan (containing 0.1–1% polymer) was treated with 20 kHz ultrasound with an ultrasonic generator (US-150T, Nissei, Tokyo, Japan) for 0–100 h. After being treated with activated charcoal, the sonicated solution was passed through a paper filter (No. 101, Toyo-Roshi, Tokyo) and a membrane filter (pore-diameter: 0.45–1.0 μm, Millipore, Medford, MA, USA). The samples were obtained by precipitation with MeOH, which contained 0.1 M NaAc as precipitant. In order to remove the NaAc, the precipitate was washed with MeOH three times and freeze-dried. Pure native succinoglycan without the sonication process was unable to be prepared for this study, because it was difficult to filtrate. Therefore, five pure fragmented samples with various molecular weights designated as samples A, B, C, D, and E were used in this study. The molecular weight distribution of the samples was estimated by a size exclude chromatography (Instrument: HLC-8220 GPC, column: TSK-Gel α-M, TOSOH, Tokyo) using pullulan (Shodex standard P-82, Showa-Denko, Tokyo) as standard. The M_Z/M_W values of these samples obtained in this way varied from 1.88 to 2.70. All experiments were performed with 0.1 M aqueous NaCl of pure fragmented succinoglycan. All other chemicals used in this study were of the height purity or HPLC grade.

2.2. Static light scattering measurement

Light scattering intensity from the succinoglycan in 0.1 M aqueous NaCl at both 25 and 75°C were measured on an automatic light scattering photometer (DLS 7000, Otsuka-Denshi, Osaka) with angular range from 30 to 130°. An He–Ne laser (633 nm) was used as the light source. The instrument was calibrated by using benzene as a reference liquid at 25°C. Optical clarification of the sample solutions were made by filtration through a membrane filter (pore-diameter: 0.45–1.0 μm, Millipore) followed by centrifugation (10,000 × g) for 1 h. The central portion of the supernatant in a centrifuge tube was collected by a syringe and directly transferred into a light scattering measurement cell (inner-diameter: 25 mm, cylindrical cell). In order to verify the heat denaturing of the succinoglycan, the measurement at 75°C was performed after the sample was incubated in the cell holder for 30 min at 75°C. Weight-average molecular weight (M_w) and the second virial coefficient (A_2) were analyzed by using Berry's square root plot,

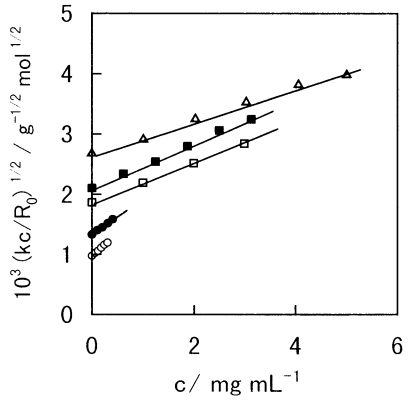

Fig. 2. Concentration dependence of $(Kc/R_0)^{1/2}$ for succinoglycan sample in 0.1 M aqueous NaCl at 25°C. The open circle represents sample A; the closed circles, sample B; the open squares, sample C; the closed squares, sample D; the open triangles, sample E. The lines indicate the regression lines of the plots.

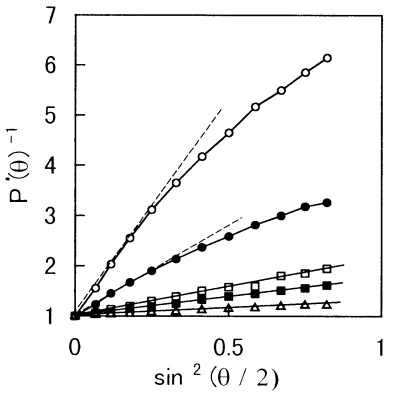

Fig. 3. Particle scattering function for the sonicated succinoglycan in 0.1 M aqueous NaCl at 25°C. The open circles represents sample A; the closed circles, sample B; the open squares, sample C; the closed squares, sample D; the open triangles, sample E. The dashed lines indicate the initial slopes of the plots.

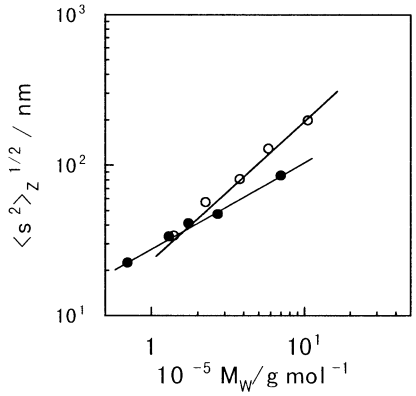

Fig. 4. Double logarithmic plots of $\langle s^2\rangle_z^{1/2}$ vs. M_w for the succinoglycan in 0.1 M aqueous NaCl at 25°C (open circles), at 75°C (closed circles).

and mean square radius of gyration ($\langle s^2\rangle_z^{1/2}$) was analyzed by using a Zimm plot.

The d*n*/d*c* of the succinoglycan in 0.1 M aqueous NaCl was measured by a differential refractometer (DRM 1021, Otsuka-Denshi) at 633 nm. The d*n*/d*c* values at 25 and 75°C were 0.150 and 0.148, respectively.

2.3. Viscometry

Zero-shear intrinsic viscosities $[\eta]_0$ of the succinoglycan in 0.1 M aqueous NaCl at both 25 and 75°C were measured by a low shear capillary viscometer with four-bulbs [19]. Apparent intrinsic viscosities were obtained by using Huggins and Mead-Fuoss plots. The $[\eta]_0$ was estimated by the extrapolation of the regression line of the apparent intrinsic viscosity and the shear rate.

3. Results and discussion

3.1. Molecular weights

Figs. 2 and 3 show the concentration dependence of $(Kc/R_0)^{1/2}$ and the angular dependence of $P(\theta)^{-1}$, respectively, for succinoglycan sample in 0.1 M aqueous NaCl at 25°C. Here, K is optical constant, R_0 is the reduced scattering intensity at zero angle, $P(\theta)$ is the particle scattering function. The results of static light scattering measurements are summarized in Table 1. In order to compare the situations of the succinoglycan at 25 and 75°C, the molecular weight ratio ((M_w at 25°C)/(M_w at 75°C)) was calculated and given in the far right column of Table 1. The table shows that the molecular weight ratios of the samples were approximately two. A_2 and $\langle s^2\rangle_z^{1/2}$ suggested that the polysaccharide dissolves as a molecular in the solution at both temperatures. The results indicate that the polysaccharide is dissolved as a dimer in water at 25°C. Here, we define, $M_w(1)$ as molecular weight of a unimer and $M_w(2)$ as molecular weight of a dimer. If the dimers are made from two polymer chains of the same length, $M_w(2)$ must be equal to $2 \times M_w(1)$. This observation is independent from the heterogeneity of the chain length of the dimers. Thus, it is thought that the dimer of the succinoglycan consists of the same length molecules at 25°C.

3.2. Molecular weight and radius of gyration

In Fig. 4, the value of $\langle s^2\rangle_z^{1/2}$ at 25 and 75°C are plotted logarithmically against M_w. The slope of the lines for the two temperatures are distinctly different from each other. It is well known that the slope indicates a molecular shape

Table 1
Molecular characteristic parameters from static light scattering in 0.1 M aqueous NaCl of the succinoglycan at both 25 and 75°C (M_W: weight average molecular weight in g mol^{-1}; $\langle s^2\rangle_z^{1/2}$: the root mean square of radius of gyration in nm; A_2: the second virial coefficient in mL g^{-2} mol; $M_W(25)/M_W(75)$ represents the ratio of molecular weight at 25 and 75°C)

Sample	25°C			75°C			$M_w(25)/M_w(75)$
	$10^{-5}\times M_w$	$\langle s^2\rangle_z^{1/2}$	$10^4\times A_2$	$10^{-5}\times M_w$	$\langle s^2\rangle_z^{1/2}$	$10^4\times A_2$	
A	10.5	199	8.18	6.97	85.8	12.9	1.5
B	5.79	129	8.75	2.70	47.6	12.7	2.1
C	3.77	81.3	10.3	1.74	41.4	14.1	2.2
D	2.25	57.1	10.4	1.30	33.8	17.6	1.7
E	1.40	34.2	9.37	0.70	22.6	22.7	2.0

Table 2
Intrinsic viscosity and Huggins constant of the succinoglycan in 0.1 M aqueous NaCl at both 25 and 75°C ($[\eta]_0$: zero-shear intrinsic viscosity in mL g^{-1}; k': Huggins constant)

Sample	25°C		75°C	
	$10^{-2} \times [\eta]_0$	k'	$10^{-2} \times [\eta]_0$	k'
A	33.6	0.416	5.90	0.431
B	15.8	0.387	2.60	0.447
C	8.80	0.412	2.00	0.346
D	4.00	0.438	1.30	0.401
E	2.10	0.378	0.820	0.335

[20]. This result indicates that the molecular shape of the succinoglycan at 25°C is different from that at 75°C. The slopes of lines for 75 and 25°C are 0.60 and 0.98, respectively. The slope (0.98) at 25°C indicates that the polysaccharide is a rod-like polymer in 0.1 mol L^{-1} NaCl. The slope (0.60) at 75°C indicates that the polysaccharide behaves as a semi-flexible polymer. Further, it can be considered that the succinoglycan has an ordered structure which is dimer and similar to a double helix at 25°C.

3.3. Molecular weight and intrinsic viscosity

The values of $[\eta]_0$ and Huggins constant k' of all samples at both 25 and 75°C are shown in Table 2, and these $[\eta]_0$ values are plotted logarithmically against M_w (Fig. 5). The data can be put on a straight line, which is obtained by the Mark–Houwink equation (the equations for 75 and 25°C are $[\eta]_0 = 6.24 \times 10^{-4} M_w^{0.86}$ and $[\eta]_0 = 1.32 \times 10^{-5} M_w^{1.40}$, respectively). The value of k' indicates that the polysaccharide dissolves as a molecular at both 25 and 75°C. The exponents of the equations (1.40 for 25°C) indicate that the polysaccharide behaves as a rod-like polymer at 25°C. On the other hand, it behaves as a semi-flexible polymer at 75°C. The results strongly support that the succinoglycan would be a double helix in 0.1 M NaCl aqueous solution at 25°C as described above.

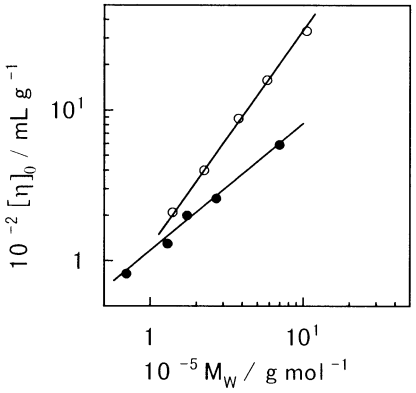

Fig. 5. Double logarithmic plots of $[\eta]_0$ vs. M_w for the succinoglycan in 0.1 M aqueous NaCl at 25°C (open circles), at 75°C (closed circles).

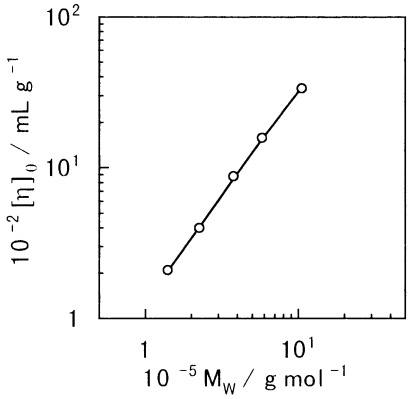

Fig. 6. Molecular weight dependence of $[\eta]$ for succinoglycan in 0.1 M aqueous NaCl at 25°C. The curve represents the theoretical values calculated from the Yamakawa–Fujii theory [21] with $q = 180$ nm, $M_L = 1510$ nm^{-1}, and $d = 4$ nm.

3.4. Worm-like chain parameters for the succinoglycan

In order to confirm the ordered structure of the polysaccharide, the linear mass density was estimated by worm-like chain model. As described in the experiment section, there is a relatively wide molecular weight distribution in our sample. Therefore, we used a hydrodynamic model that is insensitive to molecular weight distribution. Yamakawa and Fujii [21] present an equation for $[\eta]_0$ of the Kratky–Porod worm-like chain model, which is a typical hydrodynamic model for semi-flexible polymers. This equation is used for evaluating molecular characteristics for DNA [22–24], Xanthan [25,26], and Schizophyllan [27] that have multi-helical structures. The estimation using this equation was reported to suit well to the data obtained using crystallography. Therefore, the theory would be suitable to estimate the ordered conformation of the succinoglycan.

The theory of Yamakawa and Fujii for $[\eta]$ of a worm-like cylinder contains three parameters, q, M_L, and d. Here, q is persistence length, M_L is the molar mass per contour length, and d is the cylinder diameter, respectively. These three unknowns cannot uniquely be determined from our $[\eta]$ data. Since the theoretical $[\eta]$ is relatively insensitive to d, we estimated q and M_L by curve fitting for an assumed d value in the range between 3 and 5 nm. The q and M_L values obtained in this way varied from 150 to 210 nm and from 1460 to 1560 nm^{-1}, respectively. Fig. 6 shows the experimental values and the theoretical values calculated from the Yamakawa–Fujii theory with $q = 180$ nm, $M_L = 1510$ nm, and $d = 4$ nm. Since the length of the repeating unit of the succinoglycan estimated from the chemical structure is about 2 nm and the molecular weight is about 1500, the molar mass per repeating unit (M_0) should be 750 nm^{-1}. The value of M_L estimated from the experimental data at 25°C is almost twice the value of M_0 obtained from the chemical structure. Therefore, this result is strong supporting evidence for the double helical

structure of the succinoglycan at 25°C. There are no discrepancies between the results of viscometry and that of the light scattering measurement.

The q value (180 nm), is bigger than that of Xanthan (a double helix) [25,26] and is also nearly equal to that of Schizophyllan (triple helix) [27]. Borsali et al. [28] confirmed the stereoregularity and the semirigid nature of the polysaccharide by light scattering and small-angle neutron scattering. Therefore, this polysaccharide is thought to be a rigid polymer. On the other hand, the worm-like chain parameters, M_L and q at 75°C were estimated to be 750 nm^{-1} and 10 nm, respectively. The q is quite similar to that of cellulose analog [29], and the M_L is almost equal to the M_0 estimated from the chemical structure. These results give strong evidence showing that the succinoglycan dissolves as a single stranded semi-flexible polymer in 0.1 M aqueous NaCl at 75°C.

4. Conclusion

We have estimated the molecular characteristic parameters of the succinoglycan in 0.1 M aqueous NaCl at both 25 and 75°C. The molecular weight of the polysaccharide decreases to one half when the polysaccharide is heated to 75°C. The molecular weight dependence of $\langle s^2 \rangle_z$ and $[\eta]_0$ for succinoglycan in 0.1 M aqueous NaCl at both 25 and 75°C shows that the polysaccharide dissolves as a rigid rod-like polymer at 25°C and as a semi-flexible polymer at 75°C. From these data, the polysaccharide is considered to be a dimer that has ordered structure. Moreover, the molecular weight dependence of $[\eta]_0$ is explained by a worm-like chain with a persistence length of 180 nm and a linear mass density of about 1500 nm^{-1}. The latter value is almost twice that expected for single chain, so that the ordered conformation of the polysaccharide is a double helix or paired single-helices. On the other hand, molecular characteristic parameters such as A_2 and k' indicate that the polysaccharide dissolves as a molecular substance but not as an aggregate. Therefore, double helix is the most suitable candidate for the ordered conformation of the succinoglycan. However, some unclear issues remain for determination of ordered conformation of succinoglycan. As described in the introduction, there are some discrepancies between previous studies. No crystalline structure is yet known because of the low crystallinity of the polysaccharide. The study on native succinoglycan in dilute solution will show clearer findings for the ordered conformation of the polysaccharide. It will also give a clear-cut answer to the ordered structure and a molecular picture during the heat denaturing process.

References

[1] Harada T. Arch Biochem Biophys 1965;112:65–9.
[2] Zevenhuizen LPTM. J Gen Microbiol 1971;68:239–43.
[3] Zevenhuizen LPTM. Carbohydr Res 1973;26:409–19.
[4] Glasebrook J, Reed JW, Reuber TL, Walker GC. Int J Biol Macromol 1990;12:67–70.
[5] Aman D, McNeil M, Franzen LE, Darvill AG, Albersheim P. Carbohydr Res 1981;95:263–82.
[6] Harada T, Anemura A, Jansson PE, Lindberg B. Carboydr Res 1979; 77:285–8.
[7] Ullmann G, Jarry A. (Rhone-Poulenc Chimie) 1994; US Patent 5348675.
[8] Boittiaux P, Guillou V. Commun J Com Esp Deterg 1997;27:321–8.
[9] Clark-Sturman AJ, Ottelander DD, Sturla PL. ACS Symposium Series 1998;396:157–68.
[10] Jones AT, Dovle M, Davies DR. SPE Production & Facilities 1996;August:144–9.
[11] Gravanis G, Milas M, Rinaudo M, Clarke-Sturman AJ. Int J Biol Macromol 1990;12:201–6.
[12] Meade MJ, Tanebaum SW, Nakas JP. Can J Microbiol 1995;41: 1147–52.
[13] Cesaro A, Gamini A, Navarini L. Polymer 1992;33(19):4001–8.
[14] Boutebba A, Milas M, Rinaudo M. Biopolymers 1997;42(7):811–9.
[15] Gravanis G, Milas M, Rinaudo M. Int J Biol Macromol 1990;12:195–200.
[16] Balnois E, Stoll S, Wilkinson KJ, Buttle J, Rinaudo M, Milas M. Macromolecules 2000;33(20):7440–7.
[17] Burova TV, Golubeva IA, Grinberg NV, Mashkevich Aya, Grinberg Vya, Usov AI, Cesaro A. Biopolymers 1996;39:517–29.
[18] Norisuye T. Prog Polym Sci 1993;18:543–84.
[19] Einaga Y, Miyaki Y, Fujita H. J Polym Polym Phys Ed 1979;17: 2103–9.
[20] Flory PJ. Principles of polymer chemistry. Ithaca: Cornell University Press, 1953.
[21] Yamakawa H, Fujii M. Macromolecules 1974;7:128–35.
[22] Record Jr MT, Woodbury CP, Inman RB. Biopolymers 1975;14:393–408.
[23] Godfrey JE. Biophys Chem 1976;5:285–99.
[24] Jolly D, Eisenberg M. Biopolymers 1976;15:61–95.
[25] Sato T, Norisuye T, Fujita H. Polym J 1984;16:341–50.
[26] Sato T, Norisuye T, Fujita H. Macromolecules 1984;17:2696–700.
[27] Yanaki T, Norisuye T, Fujita H. Macromolecules 1980;13:1462–6.
[28] Borsali R, Rinaudo M, Noirez L. Macromolecules 1995;28(4):1085–8.
[29] Tsuboi A, Yamasaki M, Norisuye T, Teramoto A. Polym J 1995;27: 1219–29.