Article

Size-Dependent Rheological Variability of Levan Produced by *Gluconobacter Albidus* TMW 2.1191

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Abstract: Levan is a fructan-type exopolysaccharide, which is produced by many microbes from sucrose via extracellular levansucrases. The hydrocolloid properties of levan depend on its molecular weight, while it is unknown why and to which extent levan is functionally diverse in dependence of its size. The aim of our study was to get deeper insights into the size-dependent, functional variability of levan. For this purpose, levans of different sizes were produced using the water kefir isolate *Gluconobacter albidus* TMW 2.1191 and subsequently rheologically characterized. Three levan types could be identified, which are similarly branched, but significantly differ in their molecular size and rheological properties among each other. The smallest levan (< 10^7 Da) produced without adjustment of the pH exhibited Newton-like flow behavior up to a specific concentration of 25% (w/v). On the contrary, larger levans (> 10^8 Da) produced at pH ≥ 4.5 were shear-thinning and showed a gel like behavior at ≥ 5% (w/v). A third (intermediate) levan variant was obtained via production in buffers at pH 4.0 and exhibited the properties of a viscoelastic fluid at ≥ 5% (w/v). Our study reveals that the variable size and composition of levan are controllable and more decisive for its functionality than the amount of exerted levan.

Keywords: levan; *Gluconobacter*; exopolysaccharide; hydrocolloid; molecular weight; rheology

1. Introduction

Levan is a β-2,6-linked, water-soluble fructose polymer, which can be branched at position *O*1. It is produced by bacteria, archaea and fungi via secreted or cell-wall anchored, extracellular levansucrases (EC 2.4.1.10) [1,2]. These enzymes use the energy of the glycosidic bond of sucrose for fructose polymerization while glucose is continuously released. Many food-grade starter cultures like *Lactobacillus spp*. or *Gluconobacter spp*. produce levans [3-9]. Hence, levan is a natural component of sucrose containing fermented foods like sourdough breads [10], kefir [11] or natto [12,13]. Besides its prebiotic and health-promoting effects as dietary fiber [14] , high-molecular weight levan can improve the structural properties of foods [15-19] and is component of microbial biofilms [20-25]. Its functionality as hydrocolloid is mainly linked to its molecular weight, as higher molecular weight levan stronger retards bread staling [15,16]. Moreover, its size and functionality can be triggered via control of the fermentation pH during its production [19]. However, little is still known why and to which extent high molecular weight levan differs from low molecular weight levan regarding its structural and functional properties. The aim of our study was thus to produce levans of different size distributions using the kefir isolate *Gluconobacter albidus* TMW 2.1191, which encodes one

levansucrase within its genome, and to analyze the linkage types and rheological properties of the isolated levans. The obtained results should finally be correlated to get new insights into the size-dependent, functional variability of levan.

2. Material and methods

2.1. Production and recovery of levan at different pH

Gluconobacter (*G.*) *albidus* TMW 2.1191 isolated from water-kefir [7,19] was used for production and recovery of levan. Cells were generally incubated in 500 mL Erlenmeyer flasks, which were filled with 50 mL liquid medium to facilitate aerobic growth on a rotary shaker. At first, *G. albidus* was grown o/n at 200 rpm and 30 °C in liquid NaG-medium consisting of 20 g/L sodium gluconate, 3 g/L yeast extract, 2 g/L peptone, 3 g/L glycerol, 10 g/L mannitol, 3 g/L glucose (initial pH adjusted to 6.0) until an OD_{600} in the range of 2.0 - 3.0 was reached. Cells were then harvested by centrifugation (7000 x g) and resuspended in 50 mL of 0.1 M buffers (pH 3.5: citric acid/Na_2HPO_4; pH 4.0 – 5.5: Na-acetate/acetic acid; pH 6.0: Na_2HPO_4/NaH_2PO_4). For efficient levansucrase release, these buffers were additionally supplemented with 0.1 M sucrose, respectively. These suspensions were incubated for 3 h at 30 °C and 200 rpm [26,27]. Afterwards, cells were separated by centrifugation (7000 x g) and discarded. The supernatants were collected and diluted 1:1 with the same (unfermented) buffer initially used for levansucrase release (+ 0.1 M sucrose). These solutions were statically incubated for 24 h at 30 °C for production of different levan fractions. Finally, levan samples were dialyzed (MWCO: 3.5 kDa) against ddH_2O (4°C; 48 h) for removal of sugars and fructooligosaccharides < 3.5 kDa, lyophilized and weighed. For fermentative levan production without pH control, *G. albidus* was cultivated for 48 h in NaG medium containing 80 g/L sucrose as sole sugar source. After centrifugation and discarding of cells, the supernatant was treated with two volumes of chilled ethanol to precipitate the formed levan from the fermentation broth. The precipitates were collected by centrifugation (10.000 x g, 10 min, 4°C), re-dissolved in ddH_2O, dialyzed against ddH_2O (MWCO: 3.5 kDa; 4°C ; 48 h) and lyophilized. The nitrogen-contents of the levan samples isolated from NaG medium and of the levans isolated from Na-acetate buffers (pH 4.0 + 5.0) were determined using the Dumas method (DUMATHERM® CN, C. Gerhardt GmbH & Co KG, Deutschland).

2.2. Separation and size determinations of levan fractions

The molecular weights and rms radii of the recovered levans were determined by asymmetric flow field-flow fractionation (AF4; Eclipse Dualtec, Wyatt Technology, USA) coupled with multi-angle laser light scattering (MALLS) (Dawn EOS, Wyatt Technology, USA) analysis and UV detection (Dionex Ultimate 3000, Thermo Fisher Scientific, USA). The lyophilized levan was at first dissolved in ddH_2O to a final concentration of 0.1 mg/mL. 100 µl of the respective sample (10 µg) were then injected into the separation channel, equipped with a 10 kDa cellulose membrane (Nadir regenerated cellulose). Separations were performed using a detector-flow rate of 1 mL/min and a cross-flow gradient of 3 to 0.1 mL/min over 15 min, followed by 15 min of a steady cross flow of 0.1 mL/min. All chromatograms were analyzed with the software ASTRA 5 (Wyatt Technologies, Germany) using a dn/dc value of 0.146 mL/g [19] and the Berry model integrated in the ASTRA software. The extinction coefficients (λ=400 nm) of levans produced at different pH were determined in 1 mL cuvettes and a Novaspec Plus spectrophotometer (Amersham Biosciences, Germany).

2.3. Rheological measurements

Prior to rheological measurements, levan samples were dissolved in ddH_2O. Solutions with high viscosity were centrifuged for 1 min at 1000 g to remove air bubbles. Steady and dynamic rheological measurements were carried out with a Physica MCR 501 rheometer (Anton Paar, Austria) at a constant temperature of 20 °C. A double gap geometry (DG26.7-SS, Anton Paar, Austria) was used for measurements at low viscosities (levan produced in buffers with a pH ≤ 4, levan produced in buffer with a pH > 4 and ≤ 5 % (w/v), levan isolated from NaG-medium ≤ 10 % (w/v)). A cone-plate geometry (CP 50-1, Anton Paar, Austria) was used for measurements above these concentrations.

Steady shear rheological data for each levan were obtained at a concentration of 5 % (w/v). In addition, flow curves of levan produced in buffers (pH 4.0, 5,0 and 6.0) and levan isolated from NaG-medium were recorded in the concentration range between 1 % (w/v) - 10 % (w/v) and 1 % (w/v) - 25 % (w/v), respectively. The viscosity curve was measured from 0.1 s^{-1} to 1000 s^{-1} followed by a reverse sequence form 1000 s^{-1} to 0.1 s^{-1}. Fife viscosity values were recorded per order of magnitude over a period of 10 s using a logarithmic scale. In order to determine the viscoelastic properties of levan produced at pH 4.0, 5.0 and 6.0, dynamic rheological measurements were carried out at a concentration of 5 % (w/v). Small-amplitude oscillatory strain sweep experiments were performed to determine the linear visco-elastic region (data not shown). Frequency sweep tests (angular frequency 0.1 rad/s – 100 rad/s) were carried out in the linear regime, at constant strain (1.0). All rheological measurements were carried out in triplicate.

2.4. Determination of the branching degree of levan

Methylation analysis was carried out as described by Fels et al. [11] with some modifications. Briefly, levans were methylated in DMSO by using powdered sodium hydroxide and methyl iodide. Subsequently, methylated polysaccharides were recovered by extraction with dichloromethane. Due to the very low stability of fructans under acidic conditions, modified hydrolysis conditions (1 M trifluoroacetic acid, 70 °C, 30 min) were used [28]. After reduction with sodium borodeuteride and acetylation with acetic anhydride, the resulting partially methylated alditol acetates were identified by GC-MS and semiquantitatively determined by GC-FID. The FID response factors for terminal glucose, 1,3-linked glucose, and 1,3,6-linked glucose units described by Sweet et al. [29] were used for terminal fructose, 2,6-linked fructose, and 1,2,6-linked fructose units. NMR spectroscopy was carried out on an Ascend 500 MHz NMR spectrometer after dissolving the levans in D_2O.

3. Results

3.1. Amount and sizes of the produced levans

Different amounts of levan were produced in buffers of different initial pH (Fig. 1). The maximum levan amount could be recovered at pH 5.0. At pH 3.5, a significant decrease in levan production could be detected. Upon growth of *G. albidus* TMW 2.1191 in NaG medium containing 80 g/L sucrose as sole sugar source, 31.4 ± 2.3 g/L of levan could be isolated from the fermentation broth. The isolated levans were further subjected to AF4-MALLS-UV analysis (Fig. 2). The lower the pH of the used buffers, the earlier the levan fractions eluted (Fig. 2A). The levan isolated from NaG medium eluted distinctly earlier than the other levan samples. The molar masses and geometric radii of the levans were higher if the levan fractions eluted later revealing an increasing hydrodynamic volume of the levan molecules with increasing molecular weight (Fig. 2C+D). Moreover, the extinctions (λ=400 nm) of the levans increased with rising production pH and molecule size (Fig. 2B). The molar mass distribution of the levan isolated from NaG medium could not be evaluated as no usable UV concentration signals were obtained, even if a ten times higher amount of levan (100 µg) had been injected into the separation channel.

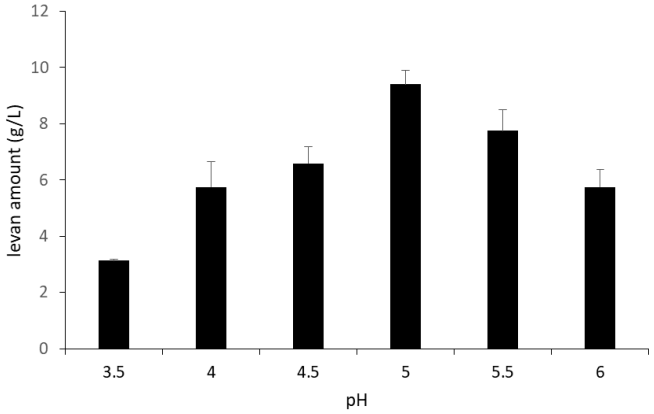

Figure 1. Levan amounts produced at different pH conditions in buffers.

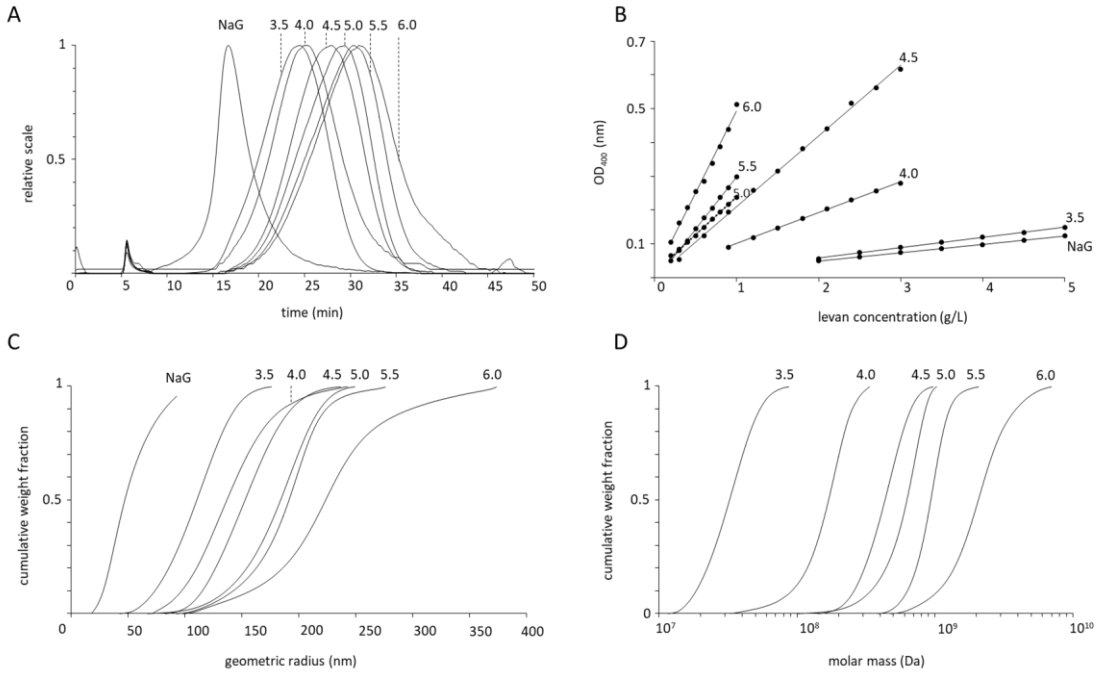

Figure 2. Elution profiles (light scattering detector 11, 90°) (A), extinction coefficients at λ = 400 nm (B), cumulative distributions of geometric radii (C) and of molar masses (D) of levans produced at different pH (3.5 – 6.0) in buffers and in NaG medium.

3.2. Rheological and structural properties of the produced levans

At first, concentration dependent flow curves were recorded for the levans produced at pH 4.0, 5.0, 6.0, and the levan isolated from NaG-medium (Fig. 3). A shear-thinning behavior was observed for levans 4.0/5.0/6.0 at specific concentrations ≥ 3% (w/v), while at 4% (w/v) the zero shear viscosity was ~ 10x higher for levans pH 5.0/6.0 than that of levan pH 4.0 (Fig. 3A-C). The viscosity (shear rate 0.1 1/s) was ~ 100x (levan concentration 5% (w/v)) and ~ 1000x higher (levan concentration 6% (w/v)) for levans produced at pH 5.0/6.0 than that of the levan produced at pH 4.0. On the contrary, no shear-thinning behavior and no distinct viscosity increase could be detected for the levan isolated from NaG medium, even if a specific concentration of 25% (w/v) had been applied (Fig. 3D). As the levan produced at pH 4.0 distinctly differed regarding its viscosity and flow behavior at 5% (w/v) from levans produced at ≥ pH 4.5 (Fig. 4A), oscillatory shear experiments were additionally performed at 5% (w/v) (Fig. 4B). Levans pH 5.0/6.0 exhibited a gel like behavior with $G'' < G'$ and a slight frequency dependence of both modules. The levan produced at a buffer pH of 4.0 showed G''

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> G´ and a steady increase of both modules over frequency. Therefore, levan pH 4.0 exhibits the properties of a viscoelastic fluid at 5% (w/v). To obtain further information about possible structural differences among the rheologically different levans, levans pH 4.0/5.0/NaG were investigated by two-dimension NMR spectroscopy and methylation analysis (Table 1). The NMR spectra were very similar for all samples and confirmed the presence of levans (data not shown). Methylation analysis allowed for the detection of 2,6-linked and 1,2,6-linked fructose units and thus linear and branched backbone units in all levan samples. The portion of terminal fructose units was rather high, however, this may be the result of some lowly abundant low molecular weight fractions or modification of levans during the course of the analysis. Nevertheless, the relative abundance of 1,2,6-linked fructose units indicates that the levans show a rather low degree of branching. In addition, the ratio between 2,6- and 1,2,6-linked fructose units (26 - 27) was almost constant for all samples, therefore, the structural analyses suggest that the all levans have a similar structural composition.

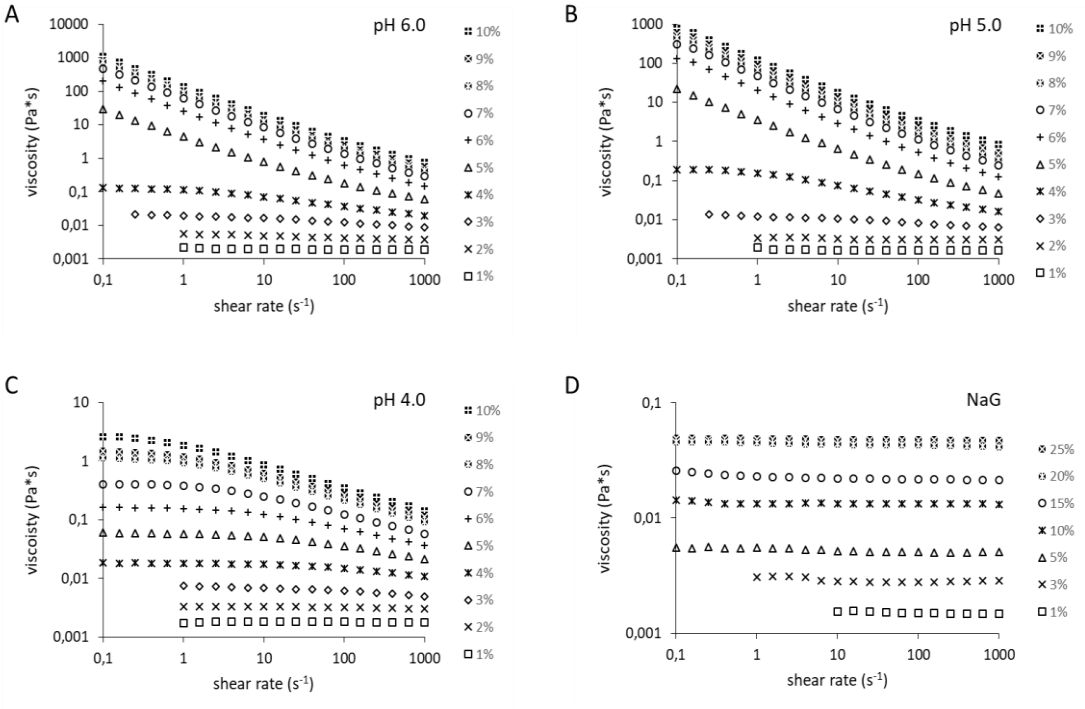

Figure 3. Concentration dependent flow curves of levans produced at pH 6.0 (A), pH 5.0 (B), pH 4.0 (C) and in NaG medium (D) recorded at 22°C, respectively.

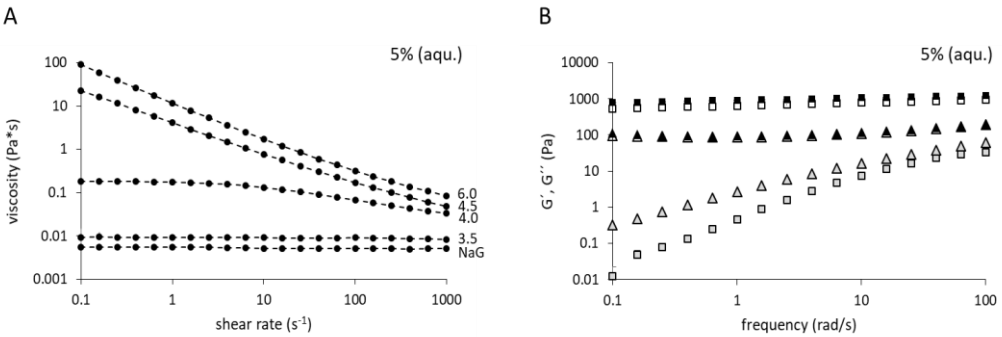

Figure 4. Flow curves of different levans produced in this study (A) and oscillatory shear measurements (B) of levans produced at pH 6.0 (black), pH 5.0 (white) and pH 4.0 (grey). Data were recorded at 22°C applying a specific concentration of 5% (aqu.), respectively. G´: square, G´´: triangles.

Table 1. Glycosidic linkages (mol%) of levans produced by G. albidus TMW 2.1191 at pH 4.0, pH 5.0, and in NaG medium as determined by methylation analysis.

Glycosidic linkage	pH 4.0	pH 5.0	NaG
t-Fru*f*	12,3 ± 3,4	13,9 ± 1,0	8,1 ± 0,4
2,6-Fru*f*	84,6 ± 3,1	83,1 ± 1,1	88,5 ± 0,3
1,2,6-Fru*f*	3,1 ± 0,3	3,1 ± 0,1	3,4 ± 0,1

Moreover, the nitrogen content of these levans (Tab. 1) was checked using a DUMAS analyzer. The nitrogen content of the levan isolated from NaG medium was slightly higher (0.053%) than that of levans pH 4.0/5.0 (0.010% - 0.016%) recovered from sodium acetate buffers.

4. Discussion

The yield, size and functional properties of levan from *G. albidus* TMW 2.1191 can be influenced by modulation of the environmental pH as demonstrated in a previous study [19]. Similar findings were obtained for the dextran produced by *Lactobacillus hordei* TMW 1.1822 [30]. The environmental pH is hence decisive to trigger the properties of uncharged, water-soluble high-molecular weight exopolysaccharides produced by glucan- or fructansucrases [31]. However, it is yet unknown to which extent the size of levan in fact influences its rheological properties. It was previously shown that levans produced by *G. albidus* TMW 2.1191 without external pH control are comparatively smaller in size at prolonged fermentation times, which might be due to (a combination of) continuous acidic hydrolysis, the intrinsic β-fructosidase activities of levansucrases or possibly additionally expressed β-fructosidases [9,19,32,33]. Accordingly, the levan recovered from NaG medium after 48 h of incubation in the present study exhibited the lowest hydrodynamic volume/radius. Moreover, Newton-like flow behavior was observed for this levan up to a specific concentration of 25% (w/v) (Fig. 3D). The levan produced in buffers at pH 3.5 also exhibited Newton-like flow behavior at 5% (w/v) despite being larger in size than levan recovered from NaG medium (Fig. 4A). Interestingly, a change of the rheological properties was detected at a specific concentration of 5% (w/v) for levan pH 4.0 (viscoelastic fluid) and levans ≥ pH 4.5 (gel like; Fig. 4 A+B).

This suggests that levan molecules produced by *Gluconobacter* and other acetic acid bacteria can interact with each other and build up intermolecular networks if a critical molecular size and a critical polymer concentration is exceeded. These networks may consist of highly entangled high molecular weight levan chains. In solutions of levan with low molecular size, the number of entanglement points between different levan chains are likely insufficient to stabilize the physical network. Therefore, these levans (pH 3.5, NaG) cannot form a physical network and show Newtonian-like behavior even at high concentrations. The levan produced at pH 4.0 exhibiting viscoelastic properties at 5% (w/v) could be considered as an intermediate between low-viscosifying (pH 3.5, NaG) and gel like levan (pH ≥ 4.5) and might have been composed of some, but too few levan molecules exhibiting the critical chain length for network formation. Therefore, the homogeneity and polydispersity of a levan fraction also contributes to its functionality. The results from the structural analyses further suggested that the degree of branching was not a deciding factor for the rheological properties in case of the analyzed levans.

A second model, which might explain the size-dependent rheological behavior of levan, is based on the secondary structure of levan in aqueous solution. With increasing molecular weight levan transforms from a random coil to a compact spherical molecule, which is increasingly compact/densely packed and turbidity forming [16,34]. Therefore, Jakob et al. [16] suggested that levan above a certain molecular weight might have the properties of a nanogel particle. Using this comparison, the viscoelastic behavior of levan of a certain size (pH ≥ 4.0) could be explained as the interaction of soft spheres forming a colloidal network [35].

5. Conclusions

Our study reveals that levan produced by the same levansucrase can differ in its rheological properties, which are rather determined by the molecular size and composition than by the exerted amount and the degree of branching of polydisperse levan. The pH used during production of levan can be adjusted to control its size and composition. These findings are a key step towards biotechnological exploitation of levan and may help to better understand its functionality in complex (food) matrices and microbial biofilms.

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Conflict of interest: The authors declare that there is no conflict of interest.

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