Carbohydrate Polymers 92 (2013) 1234–1242

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structural analysis of fructans produced by acetic acid bacteria reveals a relation to hydrocolloid function

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ARTICLE INFO

Article history:
Received 17 July 2012
Received in revised form 15 October 2012
Accepted 22 October 2012
Available online 29 October 2012

Keywords:
Acetic acid bacteria
High-molecular weight levans
Compactness
Structure
Hydrocolloid
Function

ABSTRACT

Some strains of acetic acid bacteria (*Gluconobacter frateurii* TMW 2.767, *Gluconobacter cerinus* DSM 9533T, *Neoasaia chiangmaiensis* NBRC 101099, *Kozakia baliensis* DSM 14400) produce high amounts of fructans, which can be exploited in food applications as previously demonstrated empirically for dough systems. In order to get insight into the structure and functionality of these polymers, we investigated the fructans isolated from these strains with respect to their linkage types and molecular weights/shapes using NMR spectroscopy and AF4-MALS-RI. Each fructan was identified as levan. The isolated levan fractions were highly similar according to their basic linearity and linkage types, but differed significantly in terms of their individual molecular weight distributions. In aqueous solutions the size of levan molecules present in all isolated levans continuously increased with their molecular weight and they tended to adopt a more compact molecular shape. Our data suggest that the increasing molecular weight of a levan particle enforces intramolecular interactions to reach the structural compactness of a microgel with hydrocolloid properties.

1. Introduction

Many plant, fungal and bacterial species are capable of producing fructans, which can be basically distinguished by their type of linkages (Donot, Fontana, Baccou, & Schorr-Galindo, 2012). So far, two main types of fructans have been identified: inulins (β-(2→1)-linked β-D-fructofuranosyl polymers) and levans (β-(2→6)-linked β-D-fructofuranosyl polymers) (Velazquez-Hernandez et al., 2009). Fructan synthesis is catalyzed by fructosyltransferases (Ftfs), which cleave the main substrate sucrose and release glucose in a first step. The energy derived from sucrose cleavage afterwards is used to transfer the remaining bound fructose moieties to the growing polymer chains with sucrose as starting precursor for polysaccharide formation (Jakob, Meissner, & Vogel, 2012).

Besides their biological functions as e.g. reserve carbohydrates (for plants) or protective agents against stress factors (for microorganisms; Velazquez-Hernandez et al., 2009), fructans isolated from natural sources can be applied in the industrial production of biodegradable plastics, cosmetics, glues, textile coatings and detergents (Banguela & Hernandez, 2006). Moreover, fructans and short-chain fructooligosaccharides (FOS) are considered as representative prebiotics due to their ability to preferentially stimulate the growth of intestinal bifidobacteria (Korakli, Gänzle, & Vogel, 2002; Maiorano, Piccoli, da Silva, & de Andrade Rodrigues, 2008; Roberfroid, van Loo, & Gibson, 1998; Vijn & Smeekens, 1999). Furthermore, the consumption of inulins can lead to a reduced risk of colon cancer and an enhanced calcium absorption in the gut (Coussement, 1999). Besides, it has been demonstrated that levans exhibit antitumour (Abdel-Fattah, Gamal-Eldeen, Helmy, & Esawy, 2012; Calazans, Lima, de Franca, & Lopes, 2000) and even antiviral activities (Esawy et al., 2011).

In addition to these nutritional and health-promoting aspects, especially microbial exopolysaccharides (EPSs) of high molecular weight have a high potential to improve the textural properties of foods, because they can act as thickening and gelling agents (Sutherland, 1998). Dextrans (α-(1→6)-linked glucopyranosyl polymers) and levans originating from lactobacilli can be used as bread improvers according to their ability to increase volume, retard staling and to improve texture and taste of breads (Van Geel-Schutten, 2006; Vincent et al., 2005). Hydrocolloids such as polysaccharides generally are promising compounds for these purposes due to their high water-retention capacity, which prevents extensive water loss during baking and storage of breads (Sanderson, 1996). Furthermore, polysaccharides can positively affect the pasting and rheological properties of starches and

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http://dx.doi.org/10.1016/j.carbpol.2012.10.054

stabilize freeze/thaw processes of stored breads. Xanthan, alginate, k-carrageenan, guar gum and hydroxypropylmethylcellulose (HPMC) are already applied as additives in baking applications exhibiting several of these positive functional effects (Kohajdova, Karovicova, & Schmidt, 2009).

EPSs can be isolated/produced in situ in high amounts from food grade strains (enabling clean label products), thus having the potential to replace currently used food additives (Tieking, Korakli, Ehrmann, & Vogel, 2003). Therefore, we recently screened and identified several strains of acetic acid bacteria (AAB) for/as being able to produce high amounts of polysaccharide from sucrose. AAB are already involved in several food biotechnological processes such as vinegar, bionade, kombucha or kefir production namely for their production of acetic and gluconic acid (Dufresne & Farnworth, 2000; Gulitz, Stadie, Wenning, Ehrmann, & Vogel, 2011; Kowalsky, Blum, & Weber, 2011; Pullo & Giudici, 2008). Nevertheless, their potential to produce novel EPSs for food applications had not been focused in research.

We recently demonstrated that fructans isolated from the fructan-overproducing AAB strains *Gluconobacter frateurii*, *Gluconobacter cerinus*, *Kozakia baliensis* and *Neoasaia chiangmaiensis* significantly increase volume, reduce crumb hardness and retard staling of wheat breads (Jakob, Steger, & Vogel, 2012). Furthermore, we observed different effects on the volume and texture when adding fructose polymers produced by these selected AAB species. Such empirically observed impacts of hydrocolloids like polysaccharides on food properties may be explained by the chemical structure and origin of the used additive (Rojas, Rosell, & Benedito, 1999). Thus, our objective was the establishment of a structure/function relationship referring molecule structures of fructans isolated from these AAB strains to their previously observed effects on dough systems and breads during an empirical study (Jakob, Steger, et al., 2012). Therefore, their molecular (linkage type of these polymers) and global polymer structures (molecular weight distributions and conformations/sizes) were determined and related in the discussion to the effects observed in this food system.

2. Materials and methods

2.1. Cultivation of the selected strains and EPS isolation

Gluconobacter (G.) frateurii TMW 2.767 (isolate from water kefir), *G. cerinus* DSM 9533T (isolate from cherries), *Neoasaia (N.) chiangmaiensis* NBRC 101099 (isolate from red ginger) and *Kozakia (K.) baliensis* DSM 14400 (isolate from brown palm sugar) were cultivated aerobically at 30 °C in/on modified gluconate media (Jakob, Steger, et al., 2012) (20 g/L sodium gluconate, 3 g/L yeast extract, 2 g/L peptone, 3 g/L glycerol, 10 g/L mannitol, optional 80 g/L sucrose and 20 g/L agar, pH adjusted to 6.0). For isolation of EPSs from liquid media, a single colony of the selected strain was used to inoculate 50 mL of sterile modified gluconate medium (without sucrose) and incubated for 24 h on a rotary shaker (180 rpm). After washing of the cells, 5 × 1 L of modified gluconate media containing 80 g/L sucrose were inoculated with 10 mL of the overnight culture, respectively. After aerobic cultivation of the main cultures for 48 h at 30 °C on a rotary shaker (180 rpm), cells were removed by centrifugation (5500 rpm, 10 min) and the supernatant was mixed with 2 volumes of cold ethanol and stored for 2 days at 4 °C to precipitate EPSs according to the method described by Tieking et al. (2005). The EPS precipitates did not have to be centrifuged using modified gluconate medium as they settle down at the bottom of the used precipitation flask as transparent, mucous substances. Consequently, the supernatants were discarded, the precipitates were air-dried and re-dissolved in ddH_2O. The re-dissolved EPSs were furthermore dialyzed (molecular weight cut-off: 14 kDa) against ddH_2O for at least 48 h at 4 °C to remove precipitated low molecular weight substances. Finally the purified EPSs were lyophilized.

2.2. NMR spectroscopy

In order to determine the linkage types of the isolated fructans, lyophilized EPSs were dissolved in D_2O (10 g/L). Afterwards, nuclear magnetic resonance (NMR) spectra were recorded at Bruker Avance 300 MHz at 293 K. Chemical shifts are reported in ppm (δ units) downfield from 3-(trimethylsilyl)-propionic acid-d4 (D_2O). ^{13}C NMR spectra were accumulated with a 45° pulse, 7.5/μs, 18,000 Hz spectral width, a 4 s delay between pulses and 4096 repetitions. Homonuclear shift-correlated (COSY) spectra were obtained using spectra widths of 2400 Hz and 2400 Hz, with 2 K and 128 data points in the F2 and F1 dimensions, respectively. The delay between pulses was 1.8 s and the number of repetitions 32. Heteronuclear shift-correlated two-dimensional (2D) experiments were carried out using an F2 spectral width of 12,500 Hz and an F1 width of 2400 Hz; 64 time points were accumulated with 1 K and 128 data points in F2 and F1, respectively. The delay between pulses was 1.7 s and the number of repetitions 64. Apart from the isolated EPS samples, powdered inulin from chicory roots (Sigma-Aldrich Chemie GmbH, Germany) was analyzed as described above acting as comparative control substance.

2.3. AF4-MALS-RI

Asymmetric flow field-flow fractionation (AF4) (Wyatt Technology, Germany) was used to separate fructan molecules according to the theoretical principles described by Rübsam, Krottenthaler, Gastl, and Becker (2012) and Nilsson (2012). For the determination of the corresponding molecular weights (M_Wi) and sizes of the separated molecules (radiuses of gyration, R_Gi), the AF4 system was coupled to multi-angle laser light scattering (MALS) (Dawn, Heleos II, Wyatt Technology, Germany) and a refractive index (RI) as quantitative detector (Agilent Series 1200 G1362A, Agilent Technologies, Germany). For each EPS analysis, three individual 100 μL samples (re-dissolved in ddH_2O, 1 g/L) were injected to the channel. For the separation process, the inserted spacer had a height of 350 μm and a width of 21.5 mm at the widest position approximately, at which the sample was first focused (3.5 min) in the long channel (240 mm) before it was eluted to the detectors. Measurement was carried out at 25 °C. Carrier eluent was composed of 0.05 M $NaNO_3$ and 0.003 M NaN_3 dissolved in Millipore water. Fructan molecules were separated on a 5 kDa membrane (Nadir regenerated cellulose, Wyatt Technology, Germany). Flow conditions were as follows: injection (0.2 mL/min), elution (1 mL/min) and cross flow (3 mL/min → 0.1 mL/min within 30 min). The cross flow was afterwards kept at 0.1 mL/min for 30 min and finally reduced to 0 mL/min within 18 min. The intensities of scattered light originating from the separated molecules using MALS were simultaneously measured at 18 different scattering angles (in the range of 10–160°). Concentrations of polysaccharides in aqueous solutions were determined with the coupled RI detector using a refractive index increment (dn/dc) value of 0.146 mL/g. Before analysis of the samples, the accuracy of the calibration of the analytics was verified. Pullulan standards (1 g/L, PSS GmbH, Mainz, Germany) of known molar masses were measured to prove the calibration. Blank runs were additionally performed as control to be able to subtract the resulting baselines from sample baselines. Finally, the data resulting from AF4-MALS-RI were computationally evaluated using ASTRA V 5.3.4.19 software (Wyatt Technology, Germany). Weight average (M_W) and number average (M_N) molar masses of

Table 1
^{13}C NMR chemical shifts of fructans produced by *G. frateurii*, *G. cerinus*, *N. chiangmaiensis*, *K. baliensis* and inulin from chicory roots.

Fructan	Chemical shifts (ppm) Carbon atom					
	C1	C2	C3	C4	C5	C6
G. frateurii	62.32	106.71	78.72	77.66	82.8	65.9
G. cerinus	62.71	107.08	79.11	78.04	83.17	66.26
N. chiangmaiensis	62.57	107.09	79.11	78.04	83.18	66.28
K. baliensis	62.57	106.97	78.98	77.92	83.06	66.16
Ga. xylinus	62.6[a]	106.9[a]	79.0[a]	77.9[a]	83.0[a]	66.0[a]
Inulin	63.71	106.1	79.78	77.07	83.91	64.97

[a] Data were obtained from Tajima et al. (1998).

the four different isolated fructans were calculated from the relationships

$$M_W = \frac{\sum n_i \cdot M_i^2}{\sum n_i \cdot M_i} \quad (1)$$

$$M_N = \frac{\sum n_i \cdot M_i}{\sum n_i} \quad (2)$$

where n_i represents the number of molecules with molar masses M_i.

M_W was used to calculate the average degree of polymerization *DP* of the isolated fructans following the equation

$$DP = \frac{M_W}{162.16} \quad (3)$$

where 162.16 Da is the molar mass of β-(2→6)-linked fructose in the corresponding fructan.

The quotient of M_W and M_N yields the polydispersity index (*PDI*) of the isolated polymer

$$PDI = \frac{M_W}{M_N} \quad (4)$$

Hydrodynamic coefficients ν_G of the different levan fractions were obtained from the regression lines of the log–log plots of $R_G i$ against $M_W i$ according to the relationship

$$R_G i = k_{R_G i} \cdot (M_W i)^{\nu_G} \quad (5)$$

(Nilsson, 2012), where $k_{R_G i}$ is represented as substance dependent constant.

2.4. Microscopy

Fructan isolated from *K. baliensis* was microscopically investigated using an Axiostar Plus microscope (Zeiss, Germany). Lyophilized EPS was therefore re-dissolved in ddH_2O (1 g/L), spotted on a glass slide and observed at thousandfold magnification in phase contrast mode. Digital photographs were recorded with a coupled AxioCam digital camera (ICC, Zeiss, Germany).

3. Results

3.1. NMR spectroscopy

The recorded ^{13}C NMR spectra of the isolated fructans were compared with (previously) measured chemical shift values for

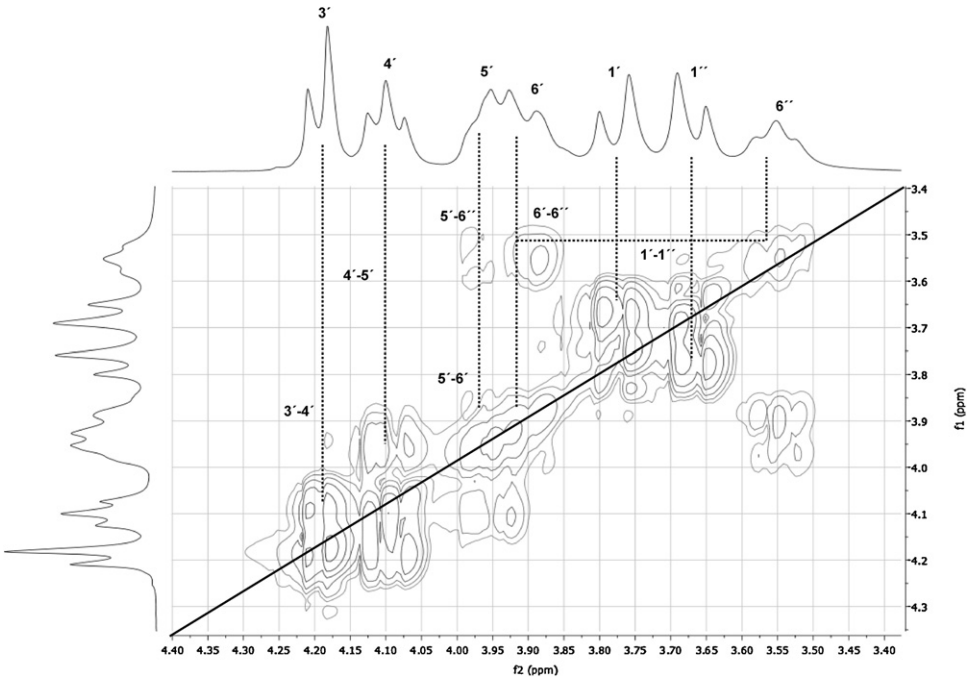

Fig. 1. ^{1}H–^{1}H 2D NMR homonuclear chemical shift correlation (COSY) spectrum of fructan isolated from *G. cerinus* DSM 9533T. Vertical dotted lines symbolize the coupling of the respective protons with their corresponding next located protons. Doublet peaks (1′-H, 1″-H, 3′-H) result from coupling with one next located proton (1′-H ↔ 1″-H, 3′-H ↔ 4′-H), triplet peaks (4′-H, 6″-H) from coupling with two next located protons (4′-H ↔ 3′-H/5′-H, 6″-H ↔ 5′-H/6′-H). The large chemical shift difference between 6′-H and 6″-H can be explained by the simultaneous shielding effects of oxygen in the fructose residue and by the glycosidic bound oxygen according to Tajima et al. (1998). The shielding effect causes an overlapping of 5′-H and 6′-H signals.

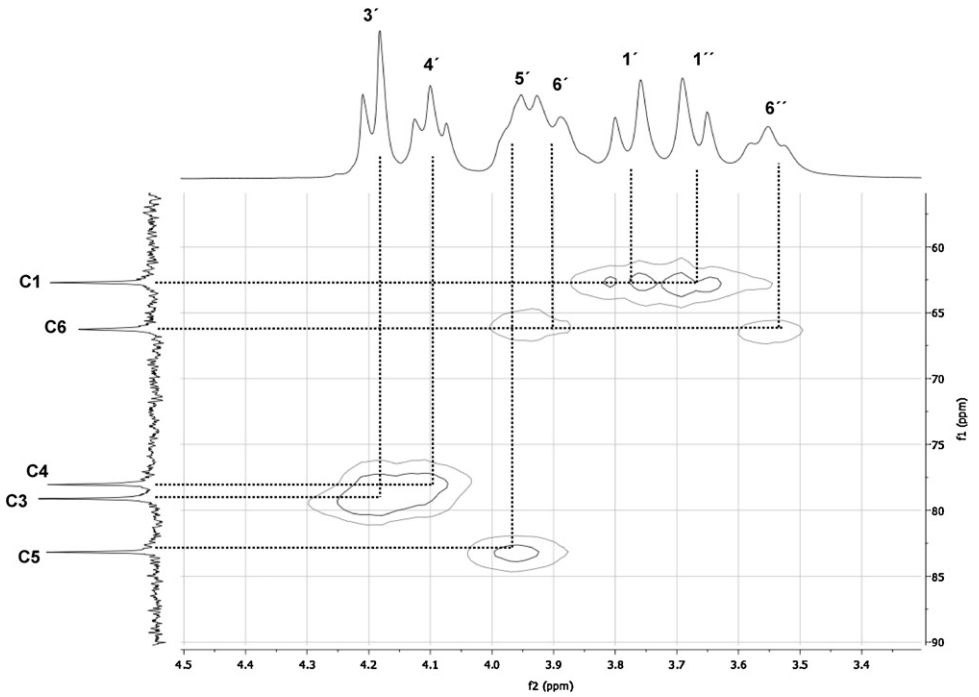

Fig. 2. ^{13}C–^{1}H 2D NMR heteronuclear multiple quantum coherence (HMQC) spectrum of fructan isolated from *G. cerinus* DSM 9533T showing attributed carbon atom signals. Dotted lines connect the corresponding carbon and hydrogen signals assigned in Fig. 1. Carbon atoms can be designated as C1–C5 according to their coupling to hydrogens. C2 (excluded for better resolution of the spectrum), which is involved in the glycosidic linkage of β-(2→6)-linked fructans and thus harbours no bound hydrogen, consequently yields no coupling signal to hydrogen (see also Fig. 1).

inulin from chicory roots and for levan from *Gluconacetobacter (Ga.) xylinus* (reclassified from the previous taxon *Acetobacter xylinum*) (Tajima et al., 1998) (Table 1). According to this, the isolated fructans of the selected AAB strains exhibited highly similar ^{13}C shift values compared to those reported for levan of *Ga. xylinus* (Tajima et al., 1998). In contrast, the ^{13}C values measured for inulin differed in a range of about 0.8–1.1 ppm from those measured for fructans from AAB strains (Table 1). Furthermore, no additional signals apart from the 6 main carbon signals could be measured in the ^{13}C-spectra of *G. frateurii*, *G. cerinus*, *N. chiangmaiensis* and *K. baliensis* indicating very few, not detectable or no carbon atoms being involved in branching. The ^{1}H NMR spectra as well as the chemical shift correlated COSY and HMQC spectra of all isolated fructans were identical as well. A small single signal at about 4.95 ppm could be observed in the anomeric regions of each of the four ^{1}H NMR spectra (data not shown), which might be due to glycosidic protons present in the terminal sucrose molecules of the respective polymer chains. Because no main signals could be detected in these anomeric regions, which potentially would result from glycosidic protons in α-/β-linked glucans, the fructan type of these polymers (no linked hydrogens present at C2 in β-(2→6) or β-(2→1) linked fructans) is confirmed. This furthermore is in agreement with the missing coupling of the corresponding C2 atoms with hydrogens derived from the recorded HMQC spectra. Moreover, two dimensional NMR (COSY, HMQC) yielded the same assignments for carbons and hydrogens present in β-(2→6)-linked fructofuranoses as reported for levan from *Ga. xylinus* (Tajima et al., 1998). Since these spectra were identical for the isolated fructans of the selected AAB strains, Figs. 1 and 2 exemplary demonstrate and explain these assignments for the fructan of *G. cerinus*. Consequently, fructans of *G. frateurii*, *G. cerinus*, *N. chiangmaiensis* and *K. baliensis* could be identified as β-(2→6)-linked β-(D)-fructofuranosyl polymers, namely levans (Fig. 3).

3.2. AF4-MALS-RI

Fig. 4 shows the cumulative distributions of obtained molecular weights (M_Wi, Fig. 4A) and molecule sizes (R_Gi, Fig. 4B) for the isolated levan polymer fractions of the four selected AAB strains. In order to investigate the continuity of molecule growth in each fraction and to describe the conformational shapes of these molecules in aqueous solution (obtained from the calculated hydrodynamic coefficients ν_G; Section 2.3), R_Gi were furthermore logarithmically plotted against their corresponding M_Wi (Fig. 5). Levan polymer fractions were divided into four overlapping M_Wi/R_Gi and three individual M_Wi/R_Gi ranges among each other to demonstrate and compare the relation of molecular weight and molecule size distributions of the investigated levans to their previously detected hydrocolloid function (Section 4.2). Table 2 summarizes M_Wi/R_Gi ranges, the percentage of molecules that were present in these ranges (calculated from data involved in Fig. 4A and B, respectively),

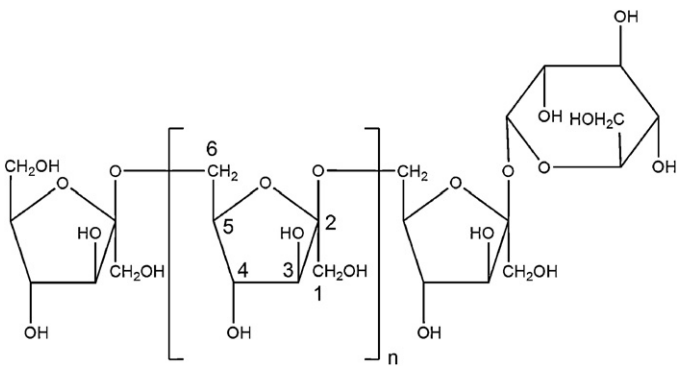

Fig. 3. Proposed structure for levans isolated from *G. frateurii*, *G. cerinus*, *N. chiangmaiensis* and *K. baliensis*.

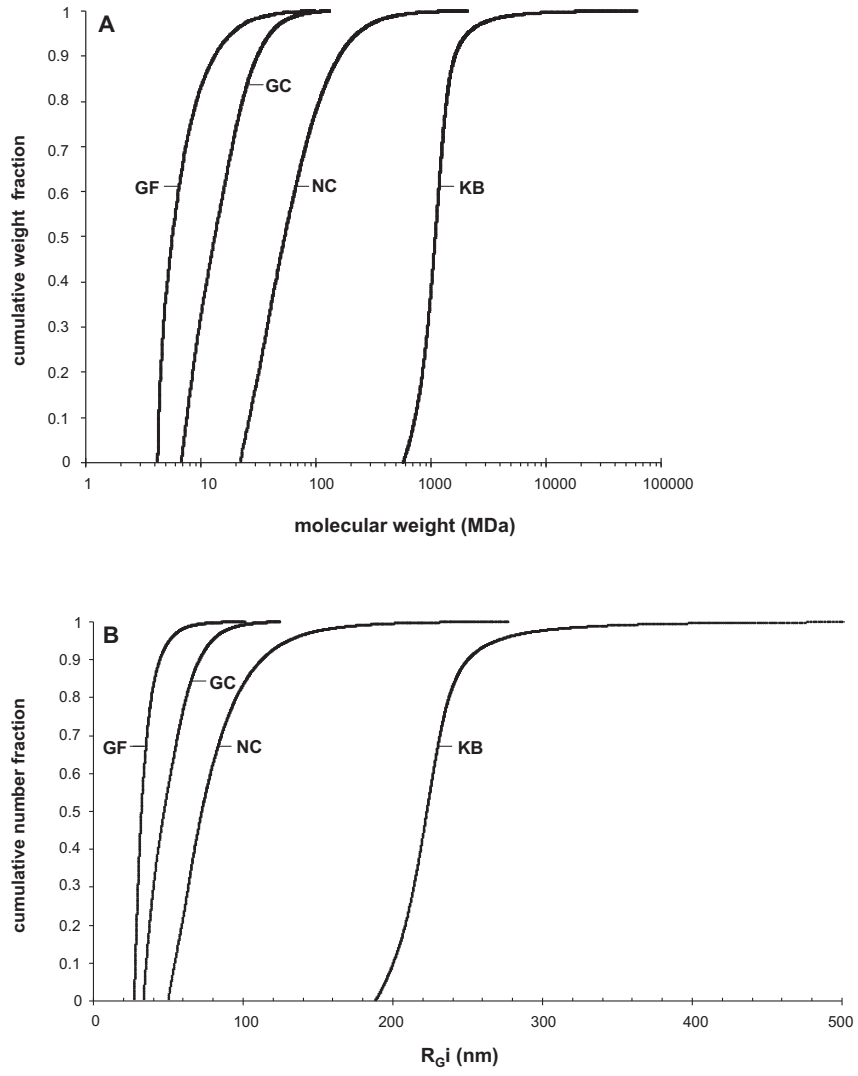

Fig. 4. Cumulative $M_W i$ (A) and $R_G i$ (B) distributions of fructans isolated from *G. frateurii* (GF), *G. cerinus* (GC), *N. chiangmaiensis* (NC) and *K. baliensis* (KB). Depicted data are mean values of three independent measurements.

and the individual hydrodynamic coefficients v_G obtained from the log–log plots of the corresponding $R_G i$ vs. $M_W i$.

In contrast to their basic structure and linkage types (Section 3.1), levan fractions isolated from the selected AAB strains significantly differed in terms of their molecular weight distributions (Fig. 4A). Whereas *G. frateurii* and *G. cerinus* synthesized polymer fractions in the range of 4–98 MDa (*G. frateurii)* and 6–98 MDa (*G. cerinus*) (100% and 99% of molecules, respectively), *N. chiangmaiensis* and *K. baliensis* produced portions of comparatively higher molecular weight levan molecules, exhibiting molecular weight ranges of 100–575 MDa (*N. chiangmaiensis*: ~20% of molecules) or even 1000–2000 MDa (*K. baliensis*: ~75% of molecules). Besides, *N. chiangmaiensis* synthesized a large fraction of molecules in the range of 22–98 MDa (77%), which was also detected to a significant lower extent in levans of *G. frateurii* (3%) and *G. cerinus* (21%). M_W/DP of the whole levan fractions of each AAB strain were calculated as 13.3 MDa/8.2×10^4 (*G. frateurii*), 26.3 MDa/1.6×10^5 (*G. cerinus*), 208.9 MDa/1.3×10^6 (*N. chiangmaiensis*) and 2466 MDa/1.5×10^7 (*K. baliensis*). The highest polydispersity index (M_W/M_N) was observed for levan from *N. chiangmaiensis* (2.51) indicating a broad distribution of molecules, which exhibited different molecular weights (see also Fig. 4A and Table 2). More narrow molecular weight distributions were detected for levans of *G. frateurii* (M_W/M_N: 1.72), *G. cerinus* (M_W/M_N: 1.53) and *K. baliensis* (M_W/M_N: 1.98).

In aqueous solutions the size of levan molecules present in all isolated levans continuously increased with their molecular weight (Figs. 4 and 5). Detected $R_G i$ value ranges in the overlapping molecular weight fractions 2, 3, 4, 6 slightly differed among the different levans (Table 2), but laid within similar $R_G i$ ranges. The hydrodynamic coefficients v_G of the whole levan fractions (calculated from all data points of each isolated fructan) were 0.44 (*G. cerinus*), 0.39 (*G. frateurii*), 0.38 (*N. chiangmaiensis*) and 0.27 (*K. baliensis*). Noticeably, the highest v_G values were calculated in the corresponding lowest molecular weight levan fractions 1 (*G. frateurii*), 2 (*G. cerinus*), 3 (*N. chiangmaiensis*) and 6 (*K. baliensis*) of each isolated levan (Table 2). Therefore, levan molecules in all isolated levans appeared to adopt a more compact conformation with increasing molecular weight (with the exception of *G. frateurii* fraction 2→3, possibly due to small detection errors, Table 2) in aqueous solution. The extraordinary high molecular weight levan molecules of *K. baliensis* could even be visualized as spherical particles using a conventional microscope at thousandfold magnification (Fig. 6).

Table 2
M_Wi/R_Gi ranges and hydrodynamic coefficients of levan fractions (according to Fig. 5) isolated from *G. frateurii* (GF), *G. cerinus* (GC), *N. chiangmaiensis* (NC) and *K. baliensis* (KB).

	Strain	Levan fraction (Fig. 5)						
		1	2	3	4	5	6	7
M_Wi range (MDa) (percentage of molecules)	GF	4–6 (64%)	6–22 (33%)	22–98 (3%)				
	GC		6–22 (78%)	22–98 (21%)	98–133 (1%)			
	NC			22–98 (77%)	98–133 (9%)	133–575 (13%)	575–2071 (1%)	
	KB						576–2069 (95%)	2069–61,637 (5%)
R_Gi range (nm) (percentage of molecules)	GF	27–34 (64%)	34–54 (33%)	54–100 (3%)				
	GC		33–61 (78%)	61–111 (21%)	111–124 (1%)			
	NC			50–92 (77%)	92–104 (9%)	104–186 (13%)	186–277 (1%)	
	KB						189–269 (95%)	269–658 (5%)
Slope of regression line (v_G)	GF	0.46	0.38	0.40				
	GC		0.52	0.40	0.34			
	NC			0.40	0.40	0.39	0.31	
	KB						0.28	0.26

Values for M_Wi/R_Gi ranges and percentages of molecules in these ranges (in parentheses) were calculated from data involved in Fig. 4. Slope values v_G were calculated from the regression lines of the different levan fractions (Fig. 5). Depicted data are mean values of three independent measurements. The coefficients of determination were >0.99 for all calculated slopes.

4. Discussion

Levan synthesis is widely spread among bacteria and was also reported for the AAB species *Gluconobacter oxydans*, *Gluconacetobacter xylinus* and *Gluconacetobacter diazotrophicus* (Velazquez-Hernandez et al., 2009). NMR structural analyses on levans from AAB are scarce, and were only performed for EPS from *Asaia bogorensis* (Kato et al., 2007) and *Ga. xylinus* (Tajima et al., 1998) yielding highly similar results as compared to the levans investigated in the current study. Recently, we have identified active levansucrases, which catalyze the formation of levans from sucrose, in *G. frateurii* TMW 2.767 and *G. cerinus* DSM 9533T (Jakob, Meissner, et al., 2012). As levansucrases could be detected in some further AAB strains so far (Jakob, Meissner, et al., 2012), levans are likely favoured EPS types for several AAB species.

4.1. Structural characterization

The isolated fructans of the selected AAB strains were identified as levans using different NMR techniques. Interestingly, the isolated levans significantly differed in terms of their molecular weight distributions. *K. baliensis* produced a levan with an M_W of 2466 MDa, which is to our knowledge the largest fructan polymer reported so far. Commercially sold high molecular weight levan was shown to have a M_W of about 900 MDa (Augsten, 2008). About 75% of levan particles from *K. baliensis* had a molecular mass in

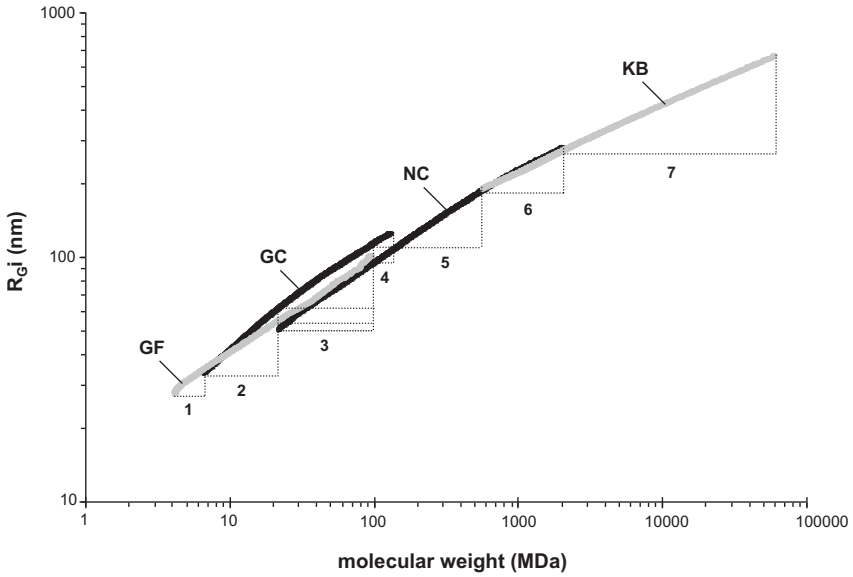

Fig. 5. Conformation plot (log–log plot of R_Gi versus M_Wi) of fructans isolated from *G. frateurii* (GF), G. *cerinus* (GC), *N. chiangmaiensis* (NC) and *K. baliensis* (KB). Depicted data are mean values of three independent measurements. Levan fractions were separated into individual (1:GF, 5:NC, 7:KB) and overlapping (2:GF/GC, 3:GF/GC/NC, 4:GC/NC, 6:NC/KB) ranges.

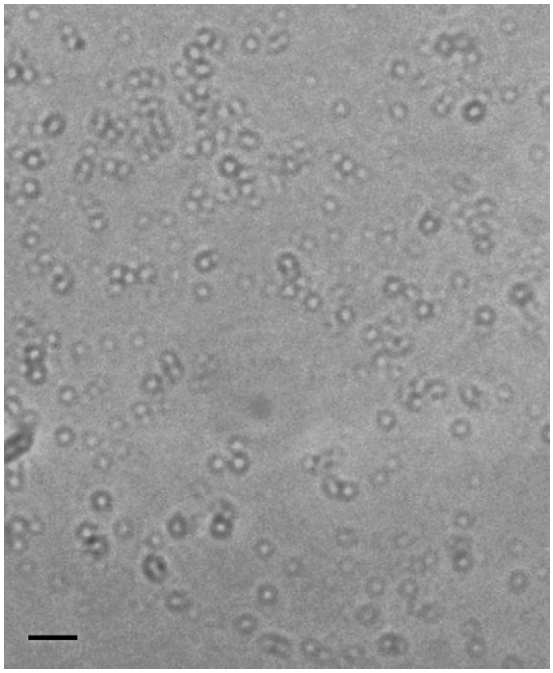

Fig. 6. Spherical shape of levan particles isolated from *K. baliensis* in aqueous solution. A solution of 1 g/L was microscopically observed at thousandfold magnification. Scale bar = 2 μm.

the range of 1000–2000 MDa (Fig. 4A). Furthermore, the hydrodynamic coefficient v_G for levan of *K. baliensis* was found to be 0.27, which indicated the highly compact structures of these molecules. Similar v_G values (derived from MALS detection) were reported for slightly branched (<5–7% β-(2→6)-linkages), in vitro synthesized high molecular weight inulins that were shown to adopt globular conformations in aqueous solutions (Wolff et al., 2000). Additional signals of carbon atoms involved in creating further linkage types could not be detected in the ^{13}C NMR spectra of the investigated levans. These levan molecules, therefore, appeared to be linear or to have too few branches to be detected using NMR spectroscopy. Similar results (no additional carbon signals in ^{13}C NMR spectra that indicate potential β-(2→1)-/β-(2→6)-linked branches in levans and inulins, respectively) were obtained for linear inulins produced by recombinant inulosucrases of *Lactobacillus gasseri* (Anwar, Kralj, van der Maarel, & Dijkhuizen, 2008).

The predicted linearity was not necessarily associated with linear conformations of these levan molecules in aqueous solution. In fact, v_G values between 0.26 (*K. baliensis*) and 0.52 (*G. cerinus*) were found in the separated levan fractions (Table 2). v_G values can be obtained from AF4-MALS using Eq. (5) (Section 2.3) to characterize the shape of a macromolecule in solution. For a sphere $v_G \sim 0.33$, for a rod v_G is close to 1 and for a random-coil macromolecule $v_G \sim 0.5$–0.6 (Nilsson, 2012). Therefore, only levan particles of relatively low molecular weight (*G. frateurii* fraction 1, *G. cerinus* fraction 2; Table 2) tended to adopt a more random coil conformation. Particles of higher molecular weight (levan fractions 3–7) tended to exhibit a more compact and spherical like one, which could even be microscopically observed in terms of the extraordinary large levan particles of *K. baliensis* (Fig. 6). These results were in agreement with previous findings reporting a spherical molecular shape of levans in aqueous solutions (Arvidson, Rinehart, & Gadala-Maria, 2006; Newbrun, Lacy, & Christie, 1971). Molecular-weight dependent conformational changes of levan (Stivala & Zweig, 1981) and inulin molecules (Kitamura et al., 1994) were observed in aqueous solutions, respectively. Polymer chains with molecular weights $<10^5$ Da exhibited random-coil conformations, whereas molecules with molar masses $>10^5$ Da adopted globular conformations. These findings were explained by strong intramolecular interactions of more distant located fructose residues among each other, which can occur at certain molecular weights. Consequently, polymer chains with critical molecular weights collapse into compact coils with an overall globular shape, in which monomers have fixed positions and in which rotations about the bonds of the backbone are severely restricted (Kitamura et al., 1994). Similar tendencies of conformational changes could be observed in the present work for each isolated levan: highest v_G values were detected in levan fractions, which were composed of polymer chains with the corresponding lowest molecular weights. Moreover, v_G values tended to decrease from lower to higher molecular weight fractions (Table 2). This again supports the findings and explanations of Stivala and Zweig (1981) and Kitamura et al. (1994) that conformations of both levan and inulin molecules in aqueous solutions strongly depend on their molecular weight. The observed increasing compactness of levan molecules in parallel with their increasing molecular weight (decreasing slope values v_G, Table 2) could furthermore be explained by strong intramolecular forces such as hydrogen bonds. These intramolecular forces could continuously increase in parallel with increasing *DP* yielding more tightly packed molecules due to more intramolecular contacts of fructose residues.

Taken together, it can be assumed, that the basic structures of all isolated levans (no detectable branches in each polymer) were highly similar among each other and that the altering molecular shapes of these molecules were primarily influenced by their molecular weight.

4.2. Relation of structural features to hydrocolloid function

The isolated levans, which were structurally examined in the present study, had been initially used for baking experiments in a former work (Jakob, Steger, et al., 2012). We observed different effects on volume, texture and shelf-life of wheat breads when adding two dosages (1% and 2% (w/w) flour) of the four different EPSs to wheat doughs. Each levan caused an increased volume, a softer crumb and a retarded staling of wheat breads during one week storage at the lowest tested dosage, demonstrating the high functional potential of these fructans. Volume increase, crumb softening and antistaling of wheat breads were significantly higher after addition of the isolated levans of *N. chiangmaiensis* and *K. baliensis* as compared to added levans of *G. frateurii* and *G. cerinus* (Jakob, Steger, et al., 2012). As shown in the current study, levans of *K. baliensis* and *N. chiangmaiensis* were composed of particles with significantly higher molecular weight and more compact molecular shape. Otherwise, levan types and proposed degrees of branching were highly similar in all investigated fructans. Consequently, higher molecular weight of these levans suggests itself as main trigger to positively influence the properties of wheat breads in terms of volume increase and retardation of staling, suggesting their hydrocolloid role in improved water retention.

Studies about the influence of different molecular weight fractions of the same polysaccharide type on the textural properties of breads are actually rare. To our knowledge, solely Skendi, Papageorgiou, and Biliaderis (2009) investigated the rheological properties of wheat doughs supplemented with β-glucans of different molecular weight, whereas the effects of β-glucan addition on finally baked breads were not determined in that work. Furthermore, only few investigations are available on the impact of fructans on wheat breads so far. Inulin was shown to exhibit limited positive technological effects on wheat and gluten-free breads (Hager et al., 2011). Though, these findings are not comparable to the present study, as the added inulin mainly consisted of short-chain oligosaccharides. The use of high molecular weight

levan produced by the sourdough strain *Lactobacillus sanfranciscensis* for preparing clean label products was patented (Vincent et al., 2005) due to its potential positive technological features such as an antistaling agent (Kaditzky, 2008). However, a structure-function relation has not been established for this levan type as well.

Regarding positive effects such as volume increase, crumb softening and antistaling of wheat breads, the chemically modified cellulose-derivative HPMC seems to be the exclusive, commonly used food hydrocolloid, that exhibits similar effects as observed for the isolated levans structurally examined in the present work (Guarda, Rosell, Benedito, & Galotto, 2004; Jakob, Steger, et al., 2012). HPMC is a good gelling agent in forming intermolecularly stabilized networks, which consist of multiple polymer chains entrapping high amounts of solvents such as water (Tritt-Goc, Kowalczuk, & Pislewski, 2005). Furthermore, this polymer was shown to enhance the hydration and water binding capacities of gluten (Barcenas, De la O-Keller, & Rosell, 2009) and proposed to inhibit starch retrogradation (Collar, Martinez, & Rosell, 2001; Guarda et al., 2004). Due to these different physicochemical properties, HPMC avoids extensive moisture loss during baking and, therefore, is supposed to soften the crumb and retard staling of wheat breads. Its ability to increase the loaf volume of breads is hardly understood and was explained by somehow increasing the gas retention during baking (Guarda et al., 2004).

Unlike HPMC, levan does not form gels from intermolecular interactions of different polymer chains in aqueous solutions and exhibits low intrinsic viscosities even at high molecular weight being typical for spherical particles (Arvidson et al., 2006). In further contrast to HPMC the levans in this study did not adopt expanded molecular structures in aqueous solutions (Wittgren & Wahlund, 1997). As described above (Section 4.1), molecular-weight dependent, conformational changes seem to be typical for both high molecular weight inulins and levans in aqueous solutions. Kitamura et al. (1994) calculated from viscosity measurements that individual high molecular weight inulin globules entrapped about 90% water within their volumes. The authors pointed out that in contrast to expanded molecules such as modified celluloses small "microgels" can be formed from individual globular inulin molecules. Consequently, the ability of the isolated levans to bind water should not result from intermolecular interactions of different elongated polymer chains. In fact, intramolecular interactions of individual levan molecules have to be considered to effectively bind water and, therefore, to act as hydrocolloids. In this context, the following considerations could differentiate the observed influences of different isolated levans on improved water retention during baking and storage of breads.

About 77% and 75% of levan particles of *N. chiangmaiensis* and *K. baliensis* had molecular weights in the range of 22–98 MDa (Table 2) and 1000–2000 MDa (calculated from data involved in Fig. 4A), respectively. Moreover, about 20% of levan particles of *N. chiangmaiensis* and *K. baliensis* had molar masses in the range of 98–575 MDa and 576–1000 MDa, respectively (Table 2). Therefore, distinctly more individual levan particles involved in EPS from *N. chiangmaiensis* were inserted into the respective doughs adding equal dosages (e.g. 10 g levan) during baking to obtain similar positive effects (see above and Jakob, Steger, et al., 2012). Consequently, the higher number of molecules with $M_W \sim 10^8$ Da (*N. chiangmaiensis*) compensated the functional effects of the comparatively lower number of molecules with $M_W \sim 10^9$ Da (*K. baliensis*) and fewer molecules of highly compact molecular shape ($\nu_G = 0.38/0.27$ for levans of *N. chiangmaiensis*/*K. baliensis*, respectively) avoided moisture loss during baking and storage similarly effective. Furthermore, the relatively low numbers of high molecular weight levan particles from *K. baliensis* and *N. chiangmaiensis* caused more positive functional effects than the comparative large number of lower molecular weight molecules of *G. frateurii* (64%: 4–6 MDa, 33%: 6–22 MDa) and *G. cerinus* (78%: 6–22 MDa, 21%: 22–98 MDa) inserted during baking using equal EPS amounts. ν_G values of the main levan fractions of *G. frateurii* (64% of molecules) and *G. cerinus* (78% of molecules) were calculated as 0.46 and 0.52, respectively (Table 2). Therefore, the main part of the isolated levan fractions from both *Gluconobacter* strains was composed of distinctly less compact particles in contrast to levans from *N. chiangmaiensis* and *K. baliensis*. Consequently, a significant proportion of levan molecules from *G. frateurii* and *G. cerinus* probably did not exhibit the efficient molecular weight/conformation for (comparatively) effective water retention. However, both levans contained enough levan particles, which were at least able to cause positive functional effects at the lowest tested dosage (Jakob, Steger, et al., 2012).

More tightly packed particles, therefore, could entrap water more effectively due to stronger intramolecular forces. Water retention hereby should have been mainly caused by levan particles acting as "microgels" themselves, rather than by interactions with flour particles such as gluten and starch. Due to its spherical shape, levan was shown to be effective in resisting interpenetration by other polymers leading to phase-separation phenomena (Kasapis, Morris, Gross, & Rudolph, 1994). As all isolated levans increased the volume of wheat breads and as levans are reported to be excellent oxygen diffusion barriers (Montana Polysaccharides Corp., www.polysaccharides.us/aboutlevan_technical.php), they could have (additionally to water) prevented extensive gas loss during baking. Still it has to be considered, that baking could induce thermal deformation or degradation of molecules. Whereas the mechanisms influenced by putatively deformed/degraded levan particles to retain water cannot be interpreted due to their unpredictable structure, it is obvious, that fewer (potentially deformed/partially degraded) particles of (initially) higher molecular weight would have made a bigger contribution for effective water retention in the complex dough system. Therefore, it could be assumed that more tightly packed levan particles were more resistant against extensive thermal deformation/degradation processes due to stronger intramolecular forces. However, Kasapis et al. (1994) could not observe any conformational changes of densely packed levan molecules during heating up to 100 °C, which cannot be surpassed in the crumb of bread as long as water is present. Taken together, our data suggest that the increasing molecular weight of a levan particle enforces intramolecular interactions to reach the structural compactness of a microgel with hydrocolloid properties.

5. Conclusions

Different species of AAB have the property to produce large amounts of structurally similar levans, some of which exhibit uniquely high molecular weights. A combination of NMR and AF4-MALS-RI techniques can be used to characterize these levan polymers in terms of linkage types and molecular weights/shapes. In this way, the present study enabled the establishment of a comparative structure-function relationship of these levans in terms of their previously observed positive functional effects on breads. This work therefore offers new possibilities to effectively adopt fructan producing, food-grade AAB strains for food applications and presents a basis to exploit the potential of these interesting polymers for further applications.

Acknowledgements

We want to thank Prof. Dr. Axel Müller (Lehrstuhl für Makromolekulare Chemie II, Universität Bayreuth) for giving us the opportunity to record the presented NMR spectra at his institute. This study was supported by funds of the innovation-promotion

program of Bundesministerium für Ernährung, Landwirtschaft und Verbraucherschutz (BMELV) through Bundesanstalt für Landwirtschaft und Ernährung (BLE) in project 28-1-63.001-07.

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