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Comparative analysis of different xanthan samples by atomic force microscopy

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ABSTRACT

The polysaccharide xanthan which is produced by the γ-proteobacterium *Xanthomonas campestris* is used as a food thickening agent and rheologic modifier in numerous food, cosmetics and technical applications. Its great commercial importance stimulated biotechnological approaches to optimize the xanthan production. By targeted genetic modification the metabolism of *Xanthomonas* can be modified in such a way that the xanthan production efficiency and/or the shear-thickening potency is optimized.

Using atomic force microscopy (AFM) the secondary structure of single xanthan polymers produced by the wild type *Xanthomonas campestris* B100 and several genetically modified variations were analyzed. We found a wide variation of characteristic differences between xanthan molecules produced by different strains. The structures ranged from single-stranded coiled polymers to branched xanthan double-strands. These results can help to get a better understanding of the polymerization- and secretion-machinery that are relevant for xanthan synthesis. Furthermore, we demonstrate that the xanthan secondary structure strongly correlates with its viscosifying properties.

1. Introduction

Xanthan, a biopolymer with outstanding rheological properties, is produced in huge amounts by fermentation of *Xanthomonas campestris* pv. campestris (Xcc). Xanthan constitutes the biggest proportion of the total world microbial polysaccharide market with an annual production exceeding 150 000 ton. Industrial scale production is done in large fermenters with a volume up to 350 000 l in a feed batch process. Xanthan is extensively used in the food-, cosmetics- and medicinal- industry but also for technical applications such as oil-drillling projects (Kreyenschulte et al., 2014). Xanthan fits well in a future-oriented market since it is produced from renewable sources. The properties of xanthan can vary significantly due to the Xcc strain from which xanthan is derived, differences in the xanthan structure and in the fermentation process such as down stream processing (Kreyenschulte et al., 2014). Besides a huge number of publications on the usage of xanthan, there is no thermodynamical model to calculate the viscosity of xanthan solutions from the chemical structure as established for hydrocarbons (Derevich, 2010).

Xanthan consists of a sugar backbone comprising two D-glucose residues with a side chain composed of D-mannose and D-glucuronic acid that are connected in a ratio of 2:2:1 (Fig. 1). Hence, a subunit of xanthan depicts a pentasaccharide (Cadmus et al., 1976). An acetyl group and a pyruvic acid substitute can be linked to the D-mannose unit which change the structure of the polymer and in case of the pyruvic acid put a negative charge on the side chain (Phillips and Williams, 2009; Hublik, 2012). These charged side chains take an important part in the building of the secondary structure of xanthan as well as in its properties in solution (Phillips and Williams, 2009).

At low physiological temperatures xanthan exists in an ordered structure which, at higher temperatures becomes disordered. This change is termed an "order-disorder transition" and depends on different factors including pH-value, pyruvate-, acetyl content and salt concentration of the solution (Arendt and Kulicke, 1998; Smith

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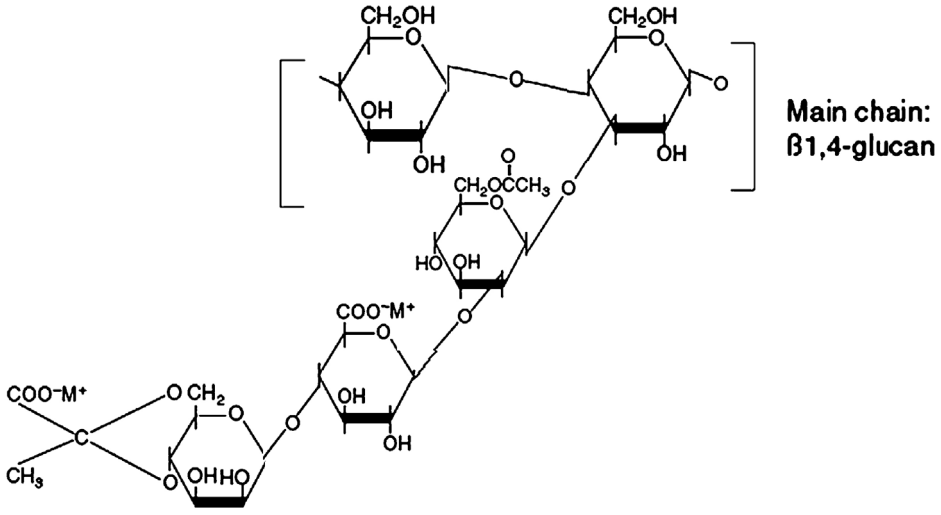

Fig. 1. Chemical structure of xanthan. D-Mannose and D-glucuronic acid units are linked to the glucose backbone. Acetate and pyruvate can be linked in different amounts to the D-mannose subunits of the chain (Hublik, 2012).

et al., 1981). It was long unclear if native xanthan exists as a single or double-stranded structure (Shatwell et al., 1990; Morris, 1998). X-ray diffraction experiments showed that xanthan exists as a right-handed helix, in which the side chains enclose the helix. However, by means of the X-ray spectra it could not be definitely deduced if the helix consists of a single xanthan polymer or if it is composed from two molecules as a double helix (Moorhouse et al., 1977; Okuyama et al., 1980). First, STM- and AFM-experiments revealed the structure and molecular conformation of commercial and wild-type xanthan (Gunning et al., 1994; Kirby et al., 1995; Gunning et al., 1996; Camesano and Wilkinson, 2001) as well as the mechanical properties of single wild-type xanthan polymers by AFM force spectroscopy (Liang et al., 2014). A new study (Moffat et al., 2016) found evidence for xanthan existing as a double-stranded helix in its ordered form. Moreover, in 2012, Gulrez et al. discovered that on positively charged mica a molecule of xanthan took the form of a 'random coil', which is less stiff and depicts the disordered form that xanthan can build in solutions with a very low ionic strength.

The synthesis of xanthan by *X. campestris* is driven by sugar nucleotides that are synthesized from phosphorylated sugars. The synthesis is mediated by enzymes encoded within a gene cluster consisting of 12 genes which code for proteins of different segments of the synthesis. The repeating unit of the xanthan polymer (Fig. 1) is synthesized bound to a C_{55}-lipid carrier at the inner leaflet of the inner membrane. The lipid carrier bearing the repeating unit is then flipped to the outer leaflet of the inner membrane and polymerized. During the polymerization the growing xanthan chain is exported to the extracellular medium. However, the exact mechanism of exportation is still unknown (Vorhoelter et al., 2008).

The main aims of our study were twofold. Firstly we wanted to find differences in the secondary structure of various xanthan samples which have been secreted by different Xcc strains. Secondly, a link between microscopic structural features of xanthan and its macroscopic viscosity should be rationalized. By means of atomic force microscopy (AFM) we analyzed several individual xanthan polymer samples of different kinds under ambient conditions and estimated structural parameters such as the molecule height, contour and persistence length. Here, we could identify characteristic secondary structures which can be attributed to the chemical composition of the xanthan side chains.

2. Experimental

2.1. Xanthan strains

Xanthan samples have been extracted from *Xanthomonas campestris* B100 cultures grown in minimal medium (Schatschneider et al., 2014) and precipitated as described by Steffens et al. (2016). Within this study we used the wild type xanthan from *X. campestris* strain B100, xanthan from an industrial production strain (JBL007; derivative of NRRL B-1459), and commercial acetate and pyruvate-free xanthan which were obtained from Jungbunzlauer (Pernhofen, Austria).

2.2. AFM imaging

All samples were available in an initial concentration of $1\,mg\,ml^{-1}$ dispensed in deionized water. Prior to AFM analysis, the samples were further diluted with deionized water in order to allow imaging of individual well separated molecules. The effective concentration varied between 2 and $10\,\mu g\,ml^{-1}$. $5\,\mu l$ of dispensed xanthan were pipetted onto freshly cleaved mica sheets and incubated for 30 s. The samples were dried under a gentle flow of dry nitrogen gas. Topographic imaging of xanthan was done using a commercial AFM (Multimode 5 and Multimode 8, Bruker, USA), respectively, using Tap300 Al-G monolithic silicon cantilevers (BudgetSensors, Bulgaria) with an aluminium reflex coating. To provide for a disturbance-free environment a vibration isolation table TS150 (TableStable LTD, Switzerland) and an accoustic enclosure (Park Systems, Korea) were used during measurements.

The scanning and imaging process was done by the AFM controller software Nanoscope (versions 5.30 and 8.15). The image resolution was set to 1280×1280 pixels for overview images ($5 \times 5\,\mu m^2$ and bigger) whereas smaller regions were scanned with a resolution of 512×512 pixels. The measurements were conducted under ambient conditions with a scan rate of 1 Hz.

2.3. Xanthan structural analysis

Raw AFM data was processed and analyzed using the Gwyddion software package (version 2.4.2) while the structural analysis was done using the software package Easyworm (Joerg Gsponer Lab,