

Altered Rheological Properties of Insoluble α -Glucan, Synthesized by Mutant Glucansucrase U13M1

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Abstract

Lactic acid bacteria from the *Leuconostoc*, *Lactobacillus*, and *Streptococcus* genera produce enzymes from the glucoside hydrolase family 70 (GH70) that synthesize α -D-glucans with various types of linkages and degrees of branching. These polymers with novel physico-chemical properties are an object of great interest for expanding application in the food, pharmaceutical, and cosmetic industries. Using site-directed mutagenesis, a mutant variant of glucansucrase URE 13-300 was obtained. The enzyme expression of the modified enzyme was optimized and the rheological properties of both types of glucans were evaluated. The modified insoluble glucan, synthesized by mutant glucansucrase U13M1 exhibited significantly higher apparent viscosity at 8°C – 3550 mPa.s at a shear rate of 0.2 s⁻¹ than the original glucan. Higher temperatures up to 80°C had a moderate influence on the viscosity as well. The presented findings are a good precondition for further exploration of modified insoluble glucans for industrial application.

Keywords: insoluble alfa-glucan, glucansucrase, *Leuconostoc*, single-point mutation, structure-rheology correlation

Резюме

Млечно-киселите бактерии от родовете *Leuconostoc*, *Lactobacillus* и *Streptococcus* продуцират ензими от семейството на гликозид хидролазите 70 (GH70), които синтезират α -D-глюкани с различни видове връзки и степен на разклоняване. Тези полимери с нови физико-химични свойства са обект на голям интерес за разширяване на приложението в хранително-вкусовата, фармацевтичната и козметичната промишленост. Използвайки сайт-насочен мутагенез, е получен мутантен вариант на глюканзахараза URE 13-300. Ензимната експресия на мутанта е оптимизирана и са оценени реологичните свойства на двата вида глюкани. Модифицираният неразтворим глюкан, синтезиран от мутантна глюканзахараза U13M1, показва значително по-висок динамичен вискозитет при 8°C – 3550 mPa.s при скорост на срязване 0.2 s⁻¹ в сравнение с оригиналния глюкан. По-високите температури до 80°C също имат умерено влияние върху измерения вискозитет. Представените изследвания са добра предпоставка за последващо разработване на модифицирани неразтворими глюкани за промишлено приложение.

Introduction

Lactic acid bacteria (LAB) are widely used in many industries because of the variety of biologically active substances they produce (Gangoiti *et al.*, 2018). Because of the probiotic status of many of them and their importance to health, they have been extensively studied. Various strains of the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Oenococcus*, *Weisella* isolated from various types of natural products such as fermented vegetables and kefir are continuously discovered (Díaz-Montes, 2021). They produce exopolysaccharides that differ in their structure and function,

as well as in the type and composition of monomers (Welman and Maddox, 2003). The enzymes synthesizing α -glucans from the glucose residues of sucrose are called glucansucrases and belong to the glycoside hydrolase family 70 (GH70), and the number of their biochemically characterized representatives has increased significantly in recent years (Moulis *et al.*, 2016; Li *et al.*, 2020). Thanks to advances in the rational design of glucansucrases to obtain polymers with desirable properties, they have the potential for multiple applications in the food, medical, and cosmetic industries as value-added products (Swistowska *et al.*, 2006; Meng *et al.*,

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2017; Claverie *et al.*, 2019). A subfamily of GH70 enzymes, called branching sucrases (BRS) are able to add single α -(1 \rightarrow 2) or α -(1 \rightarrow 3) branched glucose residues to linear dextran with mainly α -(1 \rightarrow 6) linkages as an acceptor, resulting in highly branched polysaccharides (Moulis *et al.*, 2016). The degree of branching and ratio between the different types of linkages and their organization in the polymers leads to variation in their functional properties as solubility, flow, and viscosity or conformation in solution (Li *et al.*, 2020).

Strain *Leuconostoc mesenteroides* URE 13, isolated from Bulgarian fermented vegetables, produces high molecular weight glucansucrase with a molecular mass of about 300 kDa. The gene encoding glucansucrase URE 13-300 was successfully cloned and expressed in *Escherichia coli* BL21. The synthesized glucan and oligosaccharides have a branched structure and possible prebiotic potential (Bivolarski *et al.*, 2018). The structure of the insoluble glucan, synthesized by the glucansucrase harbors a significant amount of α -(1 \rightarrow 3) linkages. Here, we present the attaining of a mutant variant of URE 13-300 enzyme via site-directed mutagenesis and the optimization of the enzyme expression. The physical properties of the glucans synthesized by the wild-type and mutant enzymes were analyzed and compared. Glucansucrases, synthesizing polymers with novel physico-chemical properties are an object of great interest in the field of applied biotechnology. The aim of the current work is to study the rheological properties of the α -glucan, synthesized by mutant glucansucrase U13M1 and the relationship to the modified structure.

Materials and Methods

Bacterial strain and plasmid vector

Recombinant strain *E. coli* BL21 URE 13-300 was used for isolation and purification of the plasmid vector, bearing the gene encoding glucansucrase URE 13-300 (using QIAprep® Miniprep Kit, Qiagen, Germany). The strain was used for enzyme production of the wild-type gene as well. It was provided by the Department of Biochemistry and Microbiology, Plovdiv University (Plovdiv, Bulgaria) (Bivolarski *et al.*, 2018).

Site-directed mutagenesis

Site-directed mutagenesis was performed with QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, USA). The following primers were designed to introduce the amino acid substitution (alternated bases are underlined): ForG449K 5'CATTAGTAGTGAGTCTAAAAAACAGAT

CATTTGCAAGGTGGTGCCTC 3'; RevG449K 5'GAGCGCACCCACCTTGCAAATGATCTGT-TTTTTTAGACTCACTACTAATG 3' (Bioneer, South Korea). PCR cycling parameters were set according to the kit instructions. After DpnI endonuclease digestion of the methylated (parental) DNA template, chemically ultracompetent *E. coli* XL10-Gold cells were transformed with the plasmids, harboring the mutated gene. Mutation efficiency was checked by control mutation of pWhitescript plasmid with color screening. The substitution of the correct amino acid was confirmed via sequencing (Macrogen, South Korea).

Optimization of mutant enzyme expression

Mutated plasmid DNA was subsequently purified as stated in the previous sections and transformed into *E. coli* BL21 (DE3) for protein expression. Initially, the cells were grown on LB broth, supplemented with 30 μ g/mL kanamycin at 37°C at 250 rpm for 18 hours. Further cultivation of the recombinant cultures was performed in Terrific Broth medium (TB) (12.0 g/L tryptone, 24.0 g/L yeast extract, 9.4 g/L K₂HPO₄, 2.2 g/L KH₂PO₄; Sigma) with 30 μ g/mL kanamycin (Merck) on a rotary shaker (250 rpm) at 37°C, and were used as inoculum for glucansucrase activity screening on TB agar plates. Positive clones were selected for optimization of the of the mutated glucansucrase URE 13-300 (U13M1). The enzyme expression was analyzed at different stages of cell growth prior to addition of inducer isopropyl-b-D-thiogalactopyranoside (IPTG). The range of tested optical density at 600 nm (OD₆₀₀) was between 0.6 and 1.8. The protein expression was studied by growing the recombinant *E. coli* BL21 cells at five different temperatures: 14°C, 16°C, 18°C, 20°C and 25°C. The effect of IPTG was also evaluated, varying between 0.3 and 1.0 mM final concentration.

Glucansucrase URE 13–300 and mutant U13M1 enzyme preparations

Cultivation of *E. coli* BL21 URE-13-300 for glucansucrase production was performed as described by Bivolarski *et al.* (2018). Pelleted cells were diluted in 20 mM sodium acetate buffer, pH 5.3, and were disrupted in Nano DeBEE High-Pressure Homogenizer (BEE International, MA, USA). The cell debris was removed by centrifugation at 8000 x g for 15 min and the supernatant was used as a source of glucansucrase URE 13-300. Preparations of mutant U13M1 enzyme were performed identically.

Enzyme activity assay

Determination of the activity of the wild-type

enzyme and its mutated variant was performed as described before (Bivolarski *et al.*, 2018) by the DNS method (Miller *et al.*, 1959). One unit of glucansucrase activity is defined as the release of 1 μ mol fructose per minute at 30°C in 20 mM sodium acetate buffer, pH 5.3; 0.05 g/L CaCl₂ (Sigma) and 100 g/L of sucrose. The concentration of the released fructose was measured spectrophotometrically at 540 nm against the appropriate standard curve. Protein concentrations were assayed by the Bradford method (Bradford, 1976). All measurements of enzyme activity were performed at least in triplicate from different experiments with standard deviation (\pm SD).

In vitro glucan synthesis

Glucan synthesis by glucansucrase URE 13-300 was carried out at 30°C with 0.5 U/mL enzyme, in 20 mM Na-acetate buffer with pH 5.5 and 100 g/L sucrose. Glucansucrase U13M1 glucan was synthesized under the following conditions: 20°C with 0.5 U/mL enzyme in 20 mM Na-acetate buffer pH 6.5 and 200 g/L sucrose. Both reactions were supplemented with 0.1% (w/v) sodium azide for the prevention of bacterial growth and were performed in a rotary shaker for 24 hours (until depletion of sucrose).

The obtained α -glucan fractions were separated by precipitation with two volumes of 96% ethanol. They were purified by triple washing with dH₂O, followed by centrifugation, and were lyophilized (Labconco FreeZone 4.5, USA) for NMR analysis and rheological assays.

Rheological properties

The lyophilized polymers, synthesized by wild-type glucansucrase URE 13-300 and mutant enzyme U13M1 were dissolved in distilled water at 6% concentration and used to study the rheological properties (Li *et al.*, 2014). The apparent viscosity of wild-type glucan was measured using a viscometer (LV DV-E, Brookfield, USA) with an RV-2 spindle. The viscosity of the modified glucan U13M1 with an RV-3 spindle. Spindle speeds of 12, 20, 30, and 50 rpm were applied during 90 s, provided that the torque to rotate the spindle in the samples was between the range of 15.0% and 85.0% of the maximum torque. The effect of increasing temperatures (8, 25, 40, 60, and 80°C) at a pH value of 6.6 was evaluated. Viscosity after swelling of the U13M1 glucan was also measured (with RV-2 spindle at the same speeds). All apparent viscosity measurements were recorded in mPa.s and were performed in triplicate.

Water holding capacity

The water-holding capacity (WHC) of α -glucans synthesized by U13M1 and URE 13-300 was evaluated as described by previous reports with modifications (Ahmed *et al.*, 2013). Sample preparation was performed by dissolving 4.0 g of the sample in 150 mL of MilliQ water and stirring at room temperature to uniform dispersion. The dispersed sample was centrifuged at 8000 \times g for 30 min and the supernatant fractions were discarded. The precipitated polymers were weighed analytically. The percentage of WHC was calculated by the following equation:

$$\text{WHC, \%} = \frac{\text{weight of the sample after water absorption}}{\text{dry sample weight}} \times 100$$

NMR analysis

Structure and linkage type and distribution in the purified polysaccharides, synthesized by the wild-type and mutant enzymes were determined by nuclear magnetic resonance (NMR) spectroscopy on Avance II (Bruker, Massachusetts, USA) as described before (Li *et al.*, 2020). Samples (5 mg) were dissolved in 0.6 mL 0.5 M NaOH diluted in D₂O, because of the insoluble nature of the studied glucans. Specters were recorded at 600 MHz for ¹H NMR analyses and the temperature of the analysis was 353 K. ¹H chemical shifts (δ) were expressed in ppm by reference to the TSPA standard (δ = 0.0 ppm). The data acquisition and processing were performed using TopSpin 4.2. software. The percentages of the linkages in glucans were calculated on the basis of the relative intensities of the anomeric protons.

Results and Discussion

Optimization of the enzyme expression of mutant glucansucrase in recombinant strain E. coli BL21

Mutant enzyme production by recombinant strain *E. coli* BL21 in shake flasks was optimized by adjusting the fermentation conditions regarding several factors: temperature for cultivation, biomass accumulation prior to induction, and concentration of added inducer isopropyl- β -D-thiogalactopyranoside (IPTG) (Fig. 1).

While investigating the correlation between accumulated biomass and enzyme production, we noted that higher enzyme activities were related to higher optical density (OD) detection prior to the addition of IPTG. The induction was set to start at an OD value between 1.6 and 1.8. The induction temperatures ranging from 14°C to 25°C were tested. The optimum appeared to be 16°C which is the same as with the initial recombinant strain (Fig. 1a).

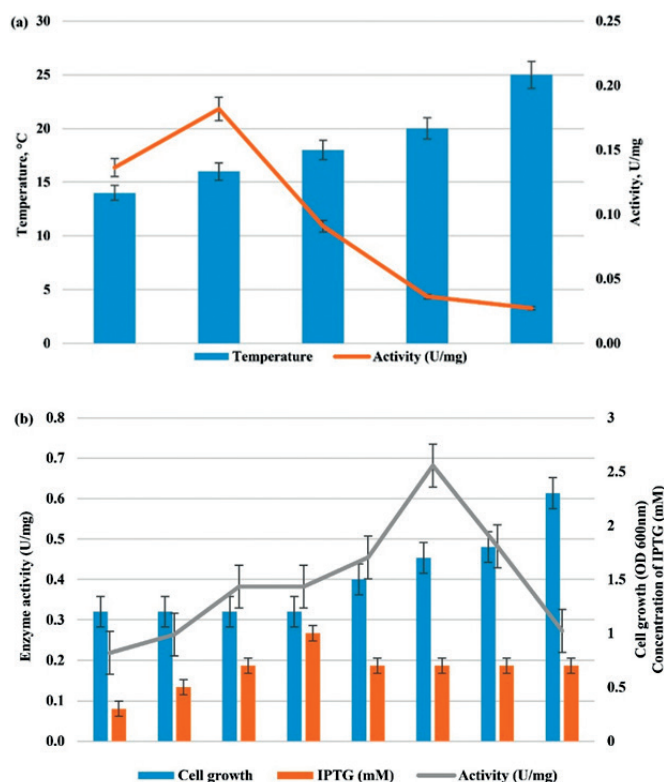


Fig. 1. Optimization of enzyme production of mutant glucanase U13M1 by cultivation of recombinant strain *E. coli* BL21-U13M1: A - optimization of the temperature for expression; B - correlation between inducer (IPTG) concentration, cell-growth prior to induction and the enzyme activity.

Further accumulation of biomass was much slower after induction and there was a notable decrease of enzyme activity, therefore the duration of fermentation was fixed at 16 hours. With respect to the amount of inductor added, the final concentration of 0.7 mM and 1.0 mM had almost the same effect (Fig. 1b). As a result of additional optimization, the highest enzyme activity of 1.3 U/mg was measured. Optimization of the mutant glucosyltransferase URE 13-300 enzyme production was essential for further examination of its functional properties. The obtained crude enzyme extracts were used for the synthesis of the glucan for investigation of its structural and functional properties.

Structural changes and water-swelling behavior of wild-type glucan and U13M1 glucan

Nuclear magnetic resonance (NMR) results obtained from the ¹H spectra of the two α -glucans showed that the ratio between the α -(1 \rightarrow 6) and α -(1 \rightarrow 3) linkages has changed due to the incorporated mutation. The polymer, synthesized by the wild-type enzyme, consists of 67.2% α -(1 \rightarrow 6) linkages and 16.2 % α -(1 \rightarrow 3) linkages. Moreover, the existing ¹H NMR signal at 4.98 ppm indicated the presence of 3,6-di-O-substituted α -D-Glcp residues

and branched structure of the analyzed glucans estimated at 8.3 % (Iliev *et al.*, 2021). The ¹H NMR spectra of the U13M1 polysaccharide revealed two anomeric protons with chemical shifts at 4.97 ppm representing 90.6 % α -(1 \rightarrow 6) linkages and 5.33 ppm, corresponding to 4.2 % α -(1 \rightarrow 3) linkages. These results show a highly reduced amount of α -(1 \rightarrow 3) linkages present in the U13M1 polymer structure. In both glucans synthesized by the wild-type and mutant enzyme, there is a signal at 5.22 and 5.25 ppm, respectively, that indicates terminal α -D-Glcp units (van Leeuwen *et al.*, 2009).

The water swelling behavior of both glucans was assessed. The water-holding capacity of U13M1 glucan was calculated to be 1125% (\pm 7.6), which is more than 1.5 times higher than the wild-type polysaccharide – 715% (\pm 6.0). Polymer samples were gradually absorbing the added water and at the end of the experiment, they were settled on the bottom of the cylinder, leaving the redundant water volume above. The insoluble polysaccharide, synthesized by U13M1 formed a more uniform suspension, however, it was not in the form of a hydrogel. High water-holding capacity is usually accompanied by high water solubility due to the absorptive structure of the polymer which can hold large quantities of water due to the formation of hydrogen bonds (Saravanan *et al.*, 2016). The exopolysaccharides from *L. lactis* KC117496 and *Lactobacillus kefiranofaciens* ZW3 have water-holding capacity values of 117% and 496 %, respectively (Ahmed *et al.*, 2013; Saravanan *et al.*, 2016). Dextran, produced by *Weissella confusa* shows similar results with 426.03% (Du *et al.*, 2022). In contrast, our results show that these insoluble glucans have even higher water-holding capacity – 715% of URE 13-300 glucan and 1125% of U13M1 glucan.

Rheological behavior of wild-type glucan and modified glucan

The apparent viscosity of the two types of glucans against increasing shear rates and at different temperatures was evaluated (Fig. 2). The apparent viscosity recorded for the original glucan has the highest value of 1980 mPa.s at a temperature of 25°C and a shear rate of 0.2 s⁻¹. Detected viscosity progressively decreased with increasing shear rate, reaching the lowest value at 750 mPa.s. This proves the non-Newtonian shear behavior of the insoluble glucan (Irague *et al.*, 2012). Increasing the temperature has a negative effect on the viscosity, reaching 623 mPa.s at 60°C. However, it was observed that the viscosity at 80°C was twice as high – 1220 mPa.s at the same shear rate 0.2 s⁻¹ (Fig. 2a).

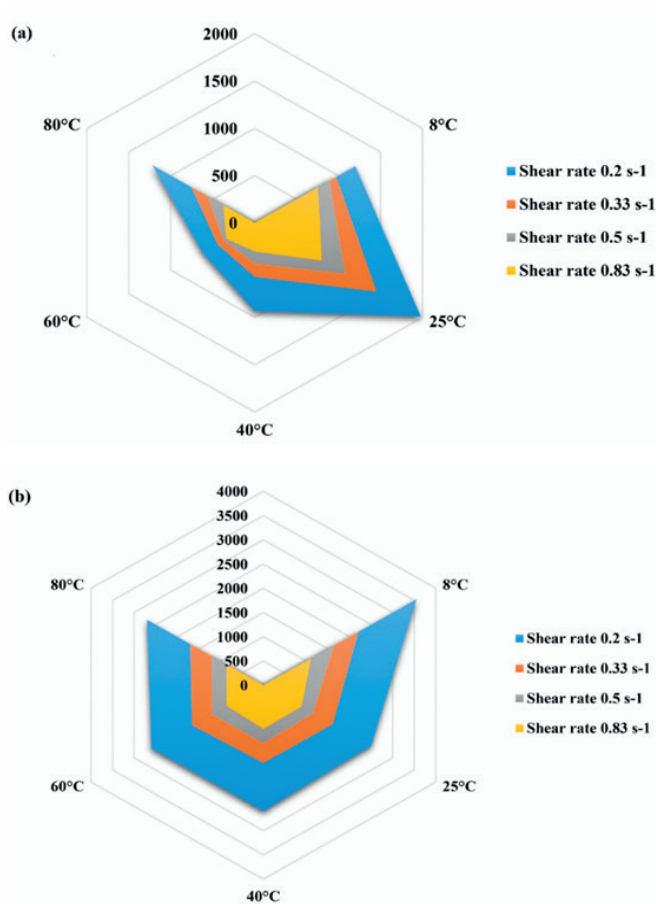


Fig. 2. Differences between the apparent viscosity of original URE 13-300 glucan (a) and U13M1 glucan (b) in relation to temperature and shear rate.

In contrast to the results obtained with the native glucan, the modified insoluble glucan synthesized by mutant glucansucrase U13M1 showed a slightly higher apparent viscosity recorded at 25°C. The differentiation is more pronounced at 8°C – 3550 mPa.s at a shear rate of 0.2 s⁻¹. Our results show that the modified polymer exhibits shear thinning behavior (decrease in apparent viscosity with increasing shear rates) as the original glucan and in accordance with DSR Δ 4N or some other previously reported dextrans (Irague *et al.*, 2012). The highest apparent viscosity of the modified glucan was recorded at the lowest temperature. Despite the apparent lower viscosity at higher temperatures, it is noted that there is no significant difference between 25°C and increasing temperatures up to 80°C (Fig 2b).

When the concentration of the modified insoluble glucan was reduced two-fold, the correlated reduction in apparent viscosity was about four-fold, from 2500 mPa.s compared to 577 mPa.s at 25°C with a shear rate of 0.2 s⁻¹. Experiments concerning the viscosity of both URE 13-300 glu-

can and U13M1 glucan showed that they exhibit shear-thinning behavior, as previously reported for other polysaccharides produced from glucansucrases (Vuillemin *et al.*, 2018). The apparent viscosity of U13M1 glucan is quite low compared to DSR-OK dextran – 2.5 Pa.s versus 2030 Pa.s (Vuillemin *et al.*, 2018). The viscosity of U13M1 glucan decreased with increasing temperature, in accordance with recent study results concerning the exopolysaccharides produced by *Lactococcus lactis* L2 (Jiang *et al.*, 2020).

Conclusion

Analyzing the acquired results from rheological assays of original glucan, synthesized by wild-type glucansucrase URE 13-300, and those of modified glucan, synthesized by mutant variant U13M1 we can conclude that there was a positive shift to higher apparent viscosity of modified polysaccharide as a result of the single-point mutation. This is a precondition for further exploration of insoluble glucans with higher viscosities and their application in the food industry, also as a protector for probiotics.

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