Exopolysaccharide from *Lactobacillus helveticus*: Identification of Chemical Structure and Effect on Biscuit Quality

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Abstract

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Exopolysaccharide (LB-gum) was produced from *Lactobacillus helveticus* by ethanol precipitation and gel-permeation chromatography. The structures of LB1 and LB2 were estimated by sugar composition analysis, methylation, and FT-IR analysis. The results proved the contents of glucose and galactose in molar ratio of 2:1 and 2.3:1, and molecular weights ~5.4 × 10^5 Da and ~20.3 × 10^5 Da, respectively. Xanthan and LB-gum were added to wheat flour during biscuits making at levels of 0, 0.5, 1.0, 1.5, and 2.0%. Rheological properties and chemical quality attributes of the biscuits during storage for six months were evaluated. The data revealed slight increases in water absorption, dough development time, and dough stability but weakening decreased about 50 %. Also, height, weight, volume, and specific volume were found to increase as xanthan and LB-gum level increased. The colour was slightly affected. There were no significant differences between the samples containing xanthan and LB-gum at different levels with respect to all parameters tested except for the taste. Shelf – life of biscuit prolonged as a result of xanthan or LB-gum addition. The best addition level to improve the biscuits quality was 1.0% of xanthan or 1.5% of LB-gum.

Keywords: exopolysaccharide; *Lactobacillus helveticus* biscuits; xanthan; LB-gum; rheological properties; chemical quality attributes; sensory evaluation

In recent years, the importance of lactic acid bacteria (LAB), both thermophilic (e.g. *Streptococcus thermophilus*) and mesophilic (*Lactococcus lactis*) strains, as producers of exopolysacchrides (EPSs) has been clearly demonstrated (De Vuyst & Degeest 1999). LAB are food grade organisms, possessing the generally-recognised status as safe (GRAS), and being able of producing EPSs that are potentially useful as thickeners, stabilisers, emulsifiers, bodying agents, gelling agents, or fat replacers in several food products (De Vuyst & Degeest 1999). Moreover, it has been suggested that EPSs produced by LAB may confer health benefits to the consumer. Some studies have indicated that these EPSs may have immunostimulatory (Hosono *et al.* 1997) and antitumoral activities (Ebina *et al.* 1995). A large biodiversity of EPSs from LAB exists , with respect to their yields, monomer compositions, molecular masses, and functionalities (Vaningelgem *et al.* 2004), the EPSs rheological properties being influenced not only by the amount of the polymer produced but also by the structure and molecular mass (Ruas-Madiedo *et al.* 2002). *Lactobacillus helveticus*, an obligately homo fer-

mentative LAB (Kandler & Weiss 1986), is able to synthesise a high molecular mass EPS (1.8 × 10^6 Da) composed of glucose and galactose (2:1) when growing in milk cultures or complex broth media containing lactose (Torino *et al.* 2005). This microorganism produces higher amounts of the polymer under acidic culture conditions (pH 4.5–5.0) as compared to the values obtained at more alkaline pH (6.2) when grown at 37°C in milk (Torino *et al*. 2001). Xanthan gum is a heteropolysaccharide produced by fermentation with the genus *Xanthomonas.* Biscuits are convenient food products and the most popular bakery items consumed nearly by all levels of society in Egypt. Some of the reasons for such wide popularity are low cost in comparison with other processed foods (affordable cost), good nutritional quality and availability of different varieties, varied taste, easy availability, and a long shelf-life. Most of the bakery products are used as a source for the incorporation of different nutritionally rich ingredients for their diversification (Sudha *et al.* 2007).

Hydrocolloids (gums) are used in starch-based products to improve stability, modify texture, facilitate processing, reduce costs, control moisture and show a variety of gelatinising and rheological properties (Krüger *et al.* 2003). Such combinations find the use in food products such as bakery and cereal products, fruit fillings, sauces, frozen foods, and confectionary products (Ward & Andon 2002). Starches and hydrocolloids are widely used in the bakery industry to impart texture and appearance properties to cereal-based foods (Ward & Andon 2002). In this investigation, we describe the isolation, fractionation, and structural features of exopolysaccharide (LB-gum) from *Lactobacillus helveticus*. In addition, the effects of LB-gum on the rheological, chemical, physical, and sensory properties as well as shelf-life of industrially produced biscuits were evaluated.

MATERIALS AND METHODS

Isolation of polysaccharide producing bacteria. Exopolysaccharide-producing bacteria were isolated by serial dilution plating on a seed media (Ronald 1997). The exopolysaccharide-producing bacteria were screened for their ability to produce exopolysaccharide, based on the colony morphology (mucous and ropy). A mucous colony was isolated and identified as *Lactobacillus helveticus.* Metabolic characterisation was carried out in Bio-Log GP2 Micro Plate™ according to the instructions of the manufacturer (BioLog, Hayward, USA) and evaluated with Microlog3 Software.

Isolation and purification of polysaccharide. *Lactobacillus helveticus* was grown in a liquid seed media under shaking conditions (150 rpm, 3 days) using the following concentrations (g/l) lactose 20.0; yeast extract 5.0; peptone from casein 5.0; K_2HPO_4 2.0; $MnSO_4 \cdot H_2O$ 0.05; $CaSO_4 \cdot 2H_2O$ 0.05; NaCl 0.2; $MgSO_4$ 0.2, and pH 5.0 (Holding & Colle 1971). The protein and cells were initially precipitated by the addition of 5% (w/v) trichloroacetic acid (TCA) to the culture; the mixture was then stirred for 4 hours. After centrifugation (5000 rpm at 4°C for 20 min), pH of the supernatant was adjusted to 7.0 with NaOH solution and the supernatant was subsequently dialysed three times (1000 ml × 3). Then cold ethanol was gradually added to the supernatant in the amount from one to two and three supernatant volumes with intermediate centrifugation. The exopolysaccharide precipitated was collected by centrifugation at 5000 rpm, washed twice with acetone and dehydrated by diethyl ether, and then dried under vacuum (Mozzi *et al.* 1996). The purity of the exopolysaccharide was examined by gel filtration chromatography using a column (16 mm × 280 mm) of Sephacryl S-200. The sample (50 mg) was dissolved in 2.0 ml of 0.05M Tris-HCl buffer (pH 7.0) and loaded onto the column. The column was eluted with the same buffer; fractions (10 ml) were collected and monitored by the phenol-H_2SO_4 method (Dubois *et al.* 1956).

Determination of molecular weight. The average molecular weights of LB1 and LB2 were determined by a gel chromatographic technique (Mozzi *et al.* 1996). Standard dextrans, 2 000 000, 500 000, and 40 000 (Fluka) were passed through a Sephacryl S-200 column (16 mm × 280 mm), and then the elution volumes were plotted against the logarithms of their respective molecular weights. A solution of the polysaccharide (3 mg) in distilled water (0.5 ml) was applied onto the column equilibrated and eluted with distilled water. The elution volume of the exopolysaccharide was then plotted in the same graph, and the molecular weights of LB1 and LB2 were determined.

Monosaccharide composition. The LB1 and LB2 were completely hydrolysed with 2M trifluoroacetic acid (TFA) at 100°C for 18 h in a boiling water bath. After the completion of hydrolysis, excess

acid was removed by co-distillation with distilled water. The hydrolysates (20 µl) were analysed by HPLC according to El-Sayed *et al.* (2005).

Infrared spectroscopy. The dried polysaccharides LB1 and LB2 were ground with KBr powder and pressed into pellets for FT-IR spectra measurement in the frequency range of 400–4000 cm^{-1} (Bruhn *et al.* 1996).

Methylation analysis of polysaccharide fractions. Permethylation of LB1 and LB2 (10 mg) was carried out using NaOH-Me_2SO-MeI (Ciucanu & Kerek 1984). This process, after the isolation of the products by neutralisation, dialysis, and evaporation, was repeated. The permethylated derivatives (2 mg) were hydrolysed with 90% HCO_2H (1 ml) at 100°C for 5 h, followed by evaporation to dryness. The residue was successively reduced with excess $NaBH_4$ and acetylated with Ac_2O-pyridine 2 ml (1:1, v/v) at room temperature for 24 h (Guilherme *et al.* 2005). The methylated alditol acetates sugars were analysed by GC-MS according to Asker *et al.* (2009).

Preparation of flour mixtures. Xanthan gum and LB-gum were added to wheat flour of 72% extraction at levels of 0%, 0.5%, 1.0%, 1.5%, and 2.0% (on flour weight basis).

Rheological properties. Rheological properties of doughs were evaluated using a Farinograph (Model Type No: 81010 (31, 50, and 63 rpm), Brabender OHG, Duisburg, Germany) according to AACC (2000).

Preparation and evaluation of biscuits. The biscuits were prepared by mixing 100 g of wheat flour of 72% extraction and xanthan gum or LB-gum at levels (0.5, 1.0, 1.5, 2.0%); 50 g of sucrose; 28 g of shortening; 0.93 g of salt; 1.11 g of sodium bicarbonate, the required volume of water, and 14.66 ml of dextrose solution (5.93%) according to the method described in (AACC 2000). The biscuits were baked in a special oven at 200°C for 15 minutes. The weight, volume, specific volume, diameter, thickness, and spread ratio of the biscuits were recorded. Organoleptic characteristics of the biscuits were evaluated with some modifications according to Zabic and Hoojjat (1984) by 10 trained panelists. The characteristics tested were the colour (10), flavour (10), taste (10), texture (10), appearance (10), and the overall acceptability (10).

Colour analysis. The colour attributes of the biscuits were evaluated using a spectro-colorimeter with CIE colour scale (Hunter, LabScan XE, Hunter Associates Laboratory, Inc., Reston, USA).

Analytical methods

Minerals content. Minerals were determined in dry ash using an atomic absorption spectrophotometer (Chapman & Pratt 1978).

Lipid autooxidation. The acid (AV) and peroxide (PV) values and thiobarbutric acid number (TBA) were the parameters used for the assessment of lipid auto-oxidation. These three parameters were determined in the oil extracted from the samples (Habib & Brown 1956) .The extracted oil was kept in a tightly closed dark bottle in a deep freezer at (–20°C) for subsequent analysis. The acid and peroxide values were determined according to the methods of AOAC (1990). Thiobarbutric acid number was determined as described by Pearson (1976).

Statistical analysis. The data of organoleptic evaluation of the biscuits were subjected to the analysis of variance and the least significant difference (LSD) at 0.05 level was calculated according to the method described by McClave and Benson (1991).

RESULTS AND DISCUSSION

Isolation and purification of exopolysaccharide (LB-gum)

The crude exopolysaccharide (LB-gum) was obtained from the culture of *Lactobacillus helveticus* by ethanol precipitation. After fractionation on Sephacryl S-200 column, LBI and LB2 were obtained (Figure 1). The average molecular weights of LB1 and LB2 were determined as ~5.4 × 10^5 Da and ~20.3 × 10^5 Da by gel permeation chromatography technique (Figure 1). The analyses of the sugars

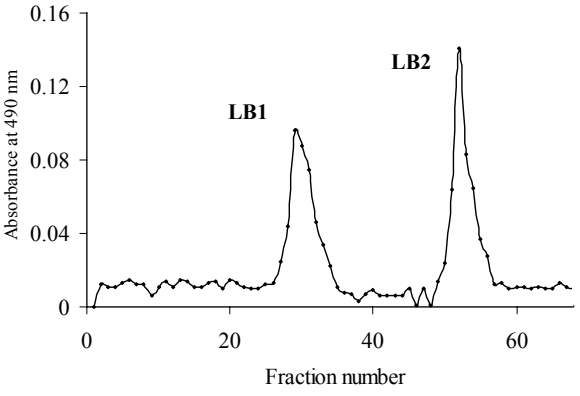

Figure1. Fractionation profile of LB-gum on the Sephacryl S-200 column

Table 1. GLC-MS data for the alditol acetates derived from methylated LB1 and LB2

Methylated sugar (as alditol acetate)	Linkage pattern	Molar ratios	
		LB1	LB2
2,3,4,6-tetra-*O*-methyl-D-glucose	Glc (1 →	0.01	0.01
2,3,6-tri-*O*-methyl-D-glucose	→ 4) Glc (1→	2.14	2.05
2,3-di-*O*-methyl-D-glucose	→ 4,6) Glc (1 →	0.08	0.09
2,3,6-tri-*O*-methyl-D-galactose	→ 4) Gal (1 →	1.00	1.00
2,3,4,6-tetra-*O*-methyl-D-galactose	Gal (1 →	0.04	0.06

compositions indicated that the polysaccharides LB1 and LB2 were mainly composed of glucose and galactose in molar ratios 2.0:1.0 and 2.3:1.0, respectively. Specterophotometrically, little or no abstraction was detected at 280 nm or at 260 nm, suggesting that the LB1 and LB2 did not content proteins or nucleic acids (Yu *et al.* 2007).

The infrared spectra of LB1 and LB2 displayed a broad stretching intense characteristic peak at around 3421 cm^{-1} for the hydroxyl group, and a weak C-H stretching band at 2923 cm^{-1}. The peak around 2165 cm^{-1} also indicated aliphatic C-H bonds. Two stretching peaks at 1084 and 1623 cm^{-1} suggest the presense of C-*O* bonds. The peak at around 898 cm^{-1} is characteristic for β-anomeric configuration (Synytsya *et al.* 2003).

The LBI and LB2 methylated products were analysed using a BD5 capillary column. LBI and LB2 showed the presence of five components, namely 2,3,4,6-tetra-*O*-methyl-D-glucose; 2,3,6-tri-*O*-methyl-D-glucose; 2,3-di-*O*-methyl-D-glucose; 2,3,6-tri-*O*-methyl-D-galactose and 2,3,4,6-tetra-*O*-methyl-D-galactose in molar ratio of 0.01:2.14:0.08:1.00:0.04 and 0.01:2.05:0.09:1.00:0.06, respectively (Table 1). The results of both infrared and methylation linkage analyses of LBI and LB2 indicated that 2,3-di-*O*-methyl-D-glucose, (1→ 4,6)-linked D-glucose and 2,3,6-tri-*O*-methyl-D-glucose (1→ 4)-linked glucose were the major components of the backbone structure with branches attached to *O*-6 of (1→ 4)-linked D-galactose. The linkage patterns of two polysaccharide fractions LBI and LB2 are similar but differ in the molecular weight only (Ray 2006; Urai *et al.* 2006).

Rheological properties of doughs as affected by added gums.

Xanthan had a very slight effect on water absorption of flour as revealed by the farinograph test. The same trend was observed as regards LB-gum a

Table 2. Farinograph parameters of dough as affected by gums

Treatments	Water absorption (%)	Arrival time (min)	Dough development time (min)	Stability (min)	Weakening (Bu)
Control	60.0	2	2	3.5	100
Xanthan					
0.5%	61.0	2.2	2.5	4.5	80
1.0%	62.0	2.5	3.0	4.0	80
1.5%	62.5	2.5	3.5	6.0	60
2.0%	63.0	2.0	3.5	8.0	40
LB-gum					
0.5%	60.0	2.0	3.0	4.0	70
1.0%	61.5	2.5	3.5	4.5	60
1.5%	61.5	3.0	3.0	5.0	55
2.0%	62.0	3.0	3.5	5.0	50

BU – Brabender units

Table 3. Baking quality of biscuits

Samples	Diameter (cm)	Height (cm)	Spread ratio (diameter/height)	Weight (g)	Volume (ml)	Specific volume (ml/g)
Control	6.2	1.10	5.64	25.29	49.86	1.97
Xanthan						
0.5%	5.8	1.20	4.83	25.76	51.70	2.00
1.0%	5.8	1.25	4.64	26.16	52.16	1.99
1.5%	5.6	1.30	4.31	26.26	53.75	2.05
2.0%	6.0	1.12	5.36	26.62	51.25	1.93
LB-gum						
0.5%	5.7	1.18	4.41	25.35	52.50	2.07
1.0%	5.7	1.22	4.67	25.50	53.75	2.11
1.5%	5.6	1.26	4.44	26.0	53.45	2.05
2.0%	5.3	1.26	4.21	26.12	53.50	2.05

slight increase was detected as concerns the arrival time and dough development time when LB-gum was added up to 2% (on flour weight basis) while no detectable effect was observed when xanthan or LB-gum were added at a level of 0.5% (Table 2). From the same table, it could be concluded that the stability of dough increased as xanthan level was increased. LB-gum effect was more pronounced than that of xanthan. The added LB-gum was highly viscous, thus being able to affect the viscoelastic properties of dough and causins an increase in the dough stability.

Baking quality of biscuits as affected by the additions of xanthan and LB-gum the data presented in Table 3 show the baking quality of the biscuits as affected by the addition of xanthan or LB-gum. Their height increased as a result of xanthan or LB-gum addition, while the diameter and spread ratio slightly decreased. The biscuit volume increased as a result of increased xanthan or LB-gum addition. The increase of the biscuit specific volume was more pronounced when xanthan or LB-gum were added at levels of 0.5% and 1%. The increasing ratio of specific volume was decreased

Table 4. Color quality of biscuits from different formulas

Samples	Crust			
	L^*	a^*	b^*	ΔE
Control	85.07	8.53	32.37	91.42
Xanthan				
0.5%	84.39	8.25	36.57	92.58
1.0%	83.66	7.75	37.41	91.97
1.5%	83.42	7.71	35.19	90.86
2.0%	82.84	7.16	35.03	90.22
LB-gum				
0.5%	85.40	7.61	33.77	92.14
1.0%	84.41	7.51	33.94	91.29
1.5%	83.62	7.37	32.83	90.13
2.0%	82.82	7.13	32.37	89.20

L^* = lightness (100 = white; 0 = black), a^* = redness (+100) to green (–80); b^* = yellowness (70) to blue (–80), $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$

Table 5. Statistical analysis of sensory properties of biscuits prepared from different Formulas

Samples	Colour (10)	Flavour (10)	Taste (10)	Texture (10)	Appearance (10)	Overall acceptability (10)
Control	8.9 ± 0.57	9.15 ± 0.58	8.5 ± 0.85[a]	8.3 ± 0.95	8.3 ± 0.63	8.0 ± 0.77
Xanthan						
0.5%	8.9 ± 0.75	9.0 ± 0.03	7.8 ± 1.14[ab]	8.0 ± 0.94	7.9 ± 0.88	8.2 ± 1.03
1.0%	9.0 ± 1.60	8.7 ± 0.95	7.6 ± 0.70[ab]	7.5 ± 0.71	8.2 ± 1.25	8.1 ± 0.79
1.5%	8.3 ± 0.95	8.7 ± 0.67	7.2 ± 1.22[bc]	7.8 ± 0.63	8.1 ± 0.76	7.8 ± 0.92
2.0%	8.9 ± 1.00	8.6 ± 0.79	7.1 ± 1.52[c]	8.0 ± 0.67	7.9 ± 0.88	7.9 ± 1.30
LB-gum						
0.5%	8.7 ± 0.99	8.5 ± 0.82	7.6 ± 1.58[ab]	7.7 ± 0.82	8.0 ± 0.67	8.4 ± 0.97
1.0%	9.0 ± 0.74	8.4 ± 0.75	8.1 ± 1.06[ab]	8.4 ± 0.75	7.9 ± 0.60	7.9 ± 0.70
1.5%	8.6 ± 0.96	8.5 ± 0.84	8.1 ± 1.06[ab]	8.1 ± 1.52	8.2 ± 1.14	7.8 ± 1.03
2.0%	8.6 ± 0.96	8.9 ± 0.67	7.8 ± 1.03[ab]	8.1 ± 1.20	7.8 ± 1.23	7.4 ± 1.43
LSD at 0.05	Ns	Ns	1.04	Ns	Ns	Ns

with high levels of addition (1.5% and 2%). Knorr (1982) stated that the addition of microcrystalline chitin increased the loaf volume of white bread and protein fortified bread.

Colour attributes of biscuits surface

The data presented in Table 4 show the colour attributes of the biscuits. In regard to the surface colour, it was clear that the addition of xanthan or LB-gum decreased the colour characteristics. As xanthan and LB-gum levels increased the lightness (L^*-value) of crust, the colour decreased, but the redness (b^*-value) of crust colour increased. That is because the presence of xanthan and LB-gum enhanced Maillard reaction, increasing red colour and darkness.

Organoleptic characteristics of the samples

The effects of xanthan and LB-gum supplementations on the organoleptic properties of the biscuits are presented in Table 5. With increasing xanthan and LB-gum levels, the sensory scores for the colour, taste, flavor, texture, appearance, and overall acceptability of the samples sharply decreased. There were no significant differences between the samples containing xanthan or LB-gum at different levels with respect to all parameters tested. Also, the results showed significant differences in taste between the samples supplemented with xanthan or LB-gum and the control.

Chemical quality attributes

The changes in fat quality parameters, i.e. AV, PV, and TBA of the biscuits prepared with either xanthan or LB-gum were followed throughout the storage period for 180 days, and the results obtained are given in Table 6. The least stable macro-components in food are the lipids. The biscuits become unacceptable and are rejected by the consumer as a result of rancidity development. To prolong the shelf-life of such foods and prevent the occurrence of rancidity, the presence of antioxidants is required. It could be noticed that AV increased gradually up to the end of the storage time in all samples. AV of the biscuits (control) increased from 0.62 mg KOH/1 g oil to 1.78 mg KOH/1 g oil at end of the storage period when xanthan and LB-gum were used, respectively. The results obtained revealed that the biscuits prepared with LB-gum had a lower AV than the control ones.

In general, the addition of antioxidants to bakery products had a good effect on decreasing the acidity of biscuits during storage, so preventing the undesirable changes in taste and flavour. Similar findings were reported by Lean and Mohamed (1999) and Mahmoud (1999). In the same Table 6, it can be noticed that PV in the samples increased during the storage period by different rates, depending on

Table 6. Effect of storage period on the chemical attributes of the stored biscuit

Treatment	Time of storage (days)											
	0			60			120			180		
	AV	PV	TBA	AV	PV	TBA	AV	PV	TBA	AV	PV	TBA
Control	0.62	3.20	0.011	1.15	10.5	0.030	1.46	15.36	0.096	1.78	20.35	0.125
Xanthan												
0.5%	0.62	3.20	0.011	1.06	8.49	0.030	1.33	10.45	0.047	1.56	16.47	0.108
1.0%	0.62	3.20	0.011	1.11	8.23	0.030	1.30	10.40	0.091	1.53	16.32	0.108
1.5%	0.62	3.20	0.011	1.07	8.25	0.029	1.33	10.36	0.091	1.51	16.32	0.107
2.0%	0.62	3.20	0.011	1.11	8.21	0.035	1.29	10.33	0.096	1.55	16.30	0.108
LB-gum												
0.5%	0.62	3.20	0.011	1.00	7.81	0.022	1.15	9.26	0.056	1.30	10.92	0.098
1.0%	0.62	3.20	0.011	0.96	7.75	0.020	1.11	9.21	0.055	1.40	10.90	0.098
1.5%	0.62	3.20	0.011	0.94	7.66	0.020	1.10	9.21	0.036	1.40	10.81	0.095
2.0%	0.62	3.20	0.011	0.93	7.50	0.019	1.12	9.00	0.035	1.36	10.78	0.093

AV – acid value (mg KOH = FFA/1 g oil); V – peroxide value (mequ peroxide/1 kg oil); TBA – thiobarbutric acid (mg malonaldehyd/1 kg oil)

the type of antioxidant used. The PV of biscuit prepared with xanthan or LB-gum increased from 3.2 at (0-time) to 16.30 and 10.78 mequ peroxide/1 kg oil (at the end of storage period), respectively. These results indicated that using antioxidants, whether xanthan or LB-gum, during processing the biscuits led to a decrease in the rate of peroxide formation in the samples during storage. These results are in agreement with those obtained by Hassanen (1998) and Mahmoud (1999). Table 6 revealed that the TBA number increased by prolongation of the storage period in both treated samples. The TBA values of the biscuits increased from 0.011 mg to 0.107 mg and 0.093 mg malonaldehyd/1 kg oil at the end of storage period with either xanthan or LB-gum addition, respectively. These results are in agreement with those obtained by Mahmoud (1999). Regarding the chemical constants (AV, PV, and TBA), it can be concluded that xanthan and LB-gum can be successfully used to retard both the oxidative and hydrolytic rancidity of biscuits and hence prolong their shelf-life.

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