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Carbohydrate Research 337 (2002) 1873–1877

CARBOHYDRATE RESEARCH

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Note

Effect of sodium orthovanadate on glycosylation of the phosphopeptidomannans involved in the cell–cell aggregation of the yeast *Kluyveromyces bulgaricus*

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Received 13 March 2002; received in revised form 7 June 2002; accepted 12 August 2002

Abstract

In studies of yeast flocculation it has been found that low concentrations of vanadium contained in sodium orthovanadate do not affect the growth and the cell–cell adhesion of the yeast *Kluyveromyces bulgaricus*, whereas high concentrations delay the growth of the yeasts and strongly inhibit flocculation. Moreover, higher sensitivity to Hygromycin B and calcofluor white was taken to imply altered cell wall integrity which is supported by compositional analysis of the extracted phosphopeptidomannans. Yeasts grown on sodium orthovanadate show a decrease in the percentage of phosphopeptidomannans and their compositions. It is proposed that the vanadium contained in sodium orthovanadate has a similar conformation to phosphorus and competes with phosphorus in phosphorylated compounds. The decrease of carbohydrate components and phosphorus linking to phosphopeptidomannans detected may alter their structure and modify ligand binding properties.

Keywords: Vanadate; Phosphopeptidomannans; Glycosylation; Cell–cell aggregation; *Kluyveromyces*

A lot of cells aggregate spontaneously. In the yeasts, this phenomenon is known as flocculation.[1] Several theories have been proposed to explain flocculation. Miki et al.[2] hypothesized that yeast flocculation involved a lectinic mechanism, this phenomenon is inhibited by specific sugars. Mannose and mannose derivatives inhibit the flocculation of *Saccharomyces cerevisiae* yeast,[3] whereas galactose and galactose derivatives inhibit the flocculation of *Kluyveromyces bulgaricus* yeast.[4] These carbohydrates inhibit the lectins which are glycoproteins,[5] recognizing the structure of glycosidic polymers, named phosphopeptidomannans (PPM), on adjacent cells.[6]

Bellal et al.[7] and Bilang et al.[8] have shown differences in PPM extracted from flocculent and non-flocculent strains which is dependant on phosphorus and competed with by vanadium. Sodium orthovanadate inhibits the formation of phosphoproteins during many important reactions[9,10] and may inhibit processes that form intermediate phosphoproteins.[11] The tetrahedron form of sodium orthovanadate has the same structure as phosphate,[12] and it has been suggested that the vanadate enzyme complex forms a trigonal bipyramidal transition state analogous to the phospho–enzyme complex formed during the intermediate state. It has been shown that the vanadate salt has toxic effects on the metabolism of *Saccharomyces cerevisiae* and a resistance mechanism was proposed.[11] Sodium orthovanadate was used to generate mutants[13,14] with defects in the N-glycosylation and O-glycosylation pathways.[15] These mutations led to altered flocculation.[15,16]

In this study we have used sodium orthovanadate to alter the glycosylation pathway of the peripheral phosphopeptidomannans and to observe its effects on the cell–cell aggregation of the yeasts *K. bulgaricus*.

Growth was not affected in the presence of 1 mM sodium orthovanadate: as in control cells, the station-

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0008-6215/02/$ - see front matter
PII: S0008-6215(02)00282-3

ary phase was reached after 20 h (Fig. 1). The lag phase increased at concentrations of 3 and 8 mM and the stationary phase was reached after 45 and 65 h of growth, respectively. The final biomass only differed by 5.2% between the cells grown in the presence of 8 mM sodium orthovanadate and other sodium orthovanadate concentrations (data not shown). Flocculation was not affected in the presence of 1 mM sodium orthovanadate. Cells that flocculated in liquid culture medium still flocculated with the same intensity in Helm's acetate buffer, whereas cells that did not flocculate in liquid culture medium did not flocculate in this buffer (data not shown).

The presence of sodium orthovanadate during growth led to the cells becoming more sensitive to Hygromycin B and calcofluor white as a function of the concentrations of sodium orthovanadate used during growth (Table 1). To confirm the effect of sodium orthovanadate on the structure of phosphopeptidomannans, we carried out a biochemical analysis of extracted PPM. Phosphorus and vanadium were analysed by the use of inductively coupled plasma (Table 2). We also showed that treatment with sodium orthovanadate affected the amino acid composition of PPM (Table 3). The most striking difference was for serine, threonine, glycine and proline.

With PPM from cells grown in 1 mM sodium orthovanadate and treated with imidazole solution, two peaks were shown by HPLC (Fig. 2), the smaller with apparent M.M. of 467 kDa was observed near to the main peak. When cells were grown in 3 mM sodium orthovanadate and treated with imidazole solution, a second small peak with an apparent M.M. of 208 kDa was observed. These peaks represent the degradation

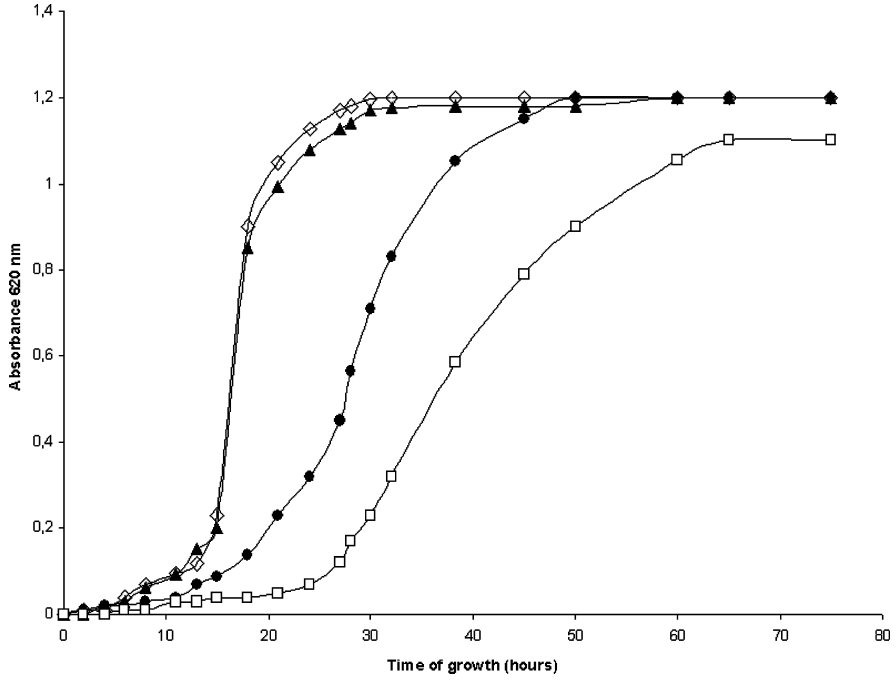

Fig. 1. Growth of *Kluyveromyces bulgaricus* in the presence of sodium orthovanadate. ––◇–– Control; ––▲–– 1 mM sodium orthovanadate; ––●–– 3 mM sodium orthovanadate; ––□–– 8 mM sodium orthovanadate.

Table 1
Effect of sodium orthovanadate on the resistance of *Kluyveromyces bulgaricus* to Hygromycin B and calcofluor white

Cell treatments	Hygromycin B μg/mL						Calcofluor white μg/mL					
	0	2	4	6	8	10	10	20	40	60	80	100
0 mM vanadate	+	+	+	+	±	±	+	+	+	±	–	–
1 mM vanadate	+	+	+	+	–	–	+	±	±	–	–	–
3 mM vanadate	+	±	±	–	–	–	+	±	±	–	–	–
8 mM vanadate	+	–	–	–	–	–	±	–	–	–	–	–

+, Growth; –, no growth.

Table 2
Biochemical analysis of phosphopeptidomannans extracted from treated and untreated cells with sodium orthovanadate

Cell treatments	PPM[a]	Carbohydrates[b]	Proteins[b]	Phosphorus[c]	Vanadium[c]	Aggregation of cells
0 mM vanadate	9.68 ± 0.75	58 ± 4	16.2 ± 0.5	0.44	0	+
1 mM vanadate	9.02 ± 0.82	54 ± 3.5	16 ± 0.22	0.42	2.54×10^{-3}	+
3 mM vanadate	1.1 ± 0.11	32.5 ± 5.2	15.8 ± 0.34	0.34	0.16	–
8 mM vanadate	0.8 ± 0.27	17.5 ± 2.8	17.5 ± 0.29	0.05	0.23	–

[a] Results are expressed in percentage of cells.
[b] Results are expressed in percentage of PPM extracted.
[c] Results are expressed in mg/mL and analysed by ICP.

Table 3
Amino acid composition of the phosphopeptidomannans extracted from treated and untreated cells with sodium orthovanadate

Amino acid in %	Cell treatments			
	0 mM vanadate	1 mM vanadate	3 mM vanadate	8 mM vanadate
Asx	16.3	16.3	11.7	15.2
Glx	17.0	17.1	17.4	19.1
Ser	6.6	6.2	5.4	3.1
Gly	5.7	6.2	8.8	11.1
His	2.0	2	1.7	0.8
Arg	3.3	3.3	2.7	0.5
Thr	9.9	7.2	5.0	3.0
Ala	7.7	8.6	9.1	14.7
Pro	1.1	1.6	4.1	8.5
Tyr	2.5	2.2	2.1	2.3
Val	4.3	4.5	4.5	4.8
Met	0.9	1.1	1.1	0.7
Ile	2.4	2.6	3.1	2.5
Leu	5.0	5.5	5.3	2.6
Phe	4.1	3.8	3.3	1.6
Lys	8.0	8.5	11.2	5.5
Cys	3.2	3.3	3.5	4.0

products of the PPM from cells treated with sodium orthovanadate. PPM extract from cells grown in the presence of 8 mM sodium orthovanadate were much less stable after treatment with imidazole solution, as shown by the numerous small peaks. Moreover, peptides with a small molecular mass result of the lack of presence of carbohydrates chains usually present on phosphopeptidomannans extract from non treated cells.

We hypothesize that carbohydrates contained in PPM are important for the exportation of PPM to the cell wall, and that this is reflector in cell wall stability. The phosphorus is substituted by vanadium, the stability of PPM from treated cells is lower than the stability of PPM extracted from untreated cells. Thus, vanadium may have pleotropic effects on the metabolism of the cell and a result of these effects is a decrease in flocculation.

1. Experimental

Microorganisms and culture conditions.—The yeast used was the flocculent *Kluyveromyces bulgaricus* (ATCC 96631) strain. Erlenmeyer flasks (100 mL) containing 20 mL of liquid culture medium (4% glucose, 0.4% peptone (primatone, Sigma), 0.1% KH_2PO_4, 0.02% $MgSO_47H_2O$, 0.02% $CaCl_2$) were sterilized at 120°C for 20 min, then inoculated with yeasts that had been maintained on Sabouraud solid medium (2% glucose, 1% peptone Primatone (Sigma), 7% agar–agar). The medium was aerated by stirring on a rotary shaker (200 rpm) for 24 h. After strong stirring to disperse the flocs, growth was monitored by measuring the absorbance at 620 nm with a Perkin Elmer spectrophotometer. The growth rate was measured according to Oberman and Libudzisz's method[17]

to determine the number of cell divisions per unit time.

Flocculation assessment.—The percentage of flocculent yeast cells was determined according to the Gilliland method[18] modified by Soares et al.[19] The following formula was applied: the percentage of flocculent yeast cells equals: A 620 $t0 - $A 620 $t7$/A 620 $t0 \times 100$.

A 620 $t0$ corresponds to the absorbance at 620 nm of the yeast suspension in the culture medium at time $t = 0$ minute, and A 620 $t7$ corresponds to the absorbance at 620 nm of the yeast suspension in the culture medium after 7 min without stirring. These experiments were also carried out in Helm's acetate buffer (150 mM CH_3COONa, 3 mM NaN_3, 3.75 mM $CaCl_2$). The yeast cells were harvested by centrifugation at 3000*g* at the end of the exponential growth phase and were washed in distilled water. Cells were suspended at a concentration of 4% in the Helm's acetate buffer and cell–cell aggregation was estimated as described before.

Toxicity assays.—We tested the effect of sodium orthovanadate on the growth and the flocculation of *K. bulgaricus*. A 1 M sodium orthovanadate solution was prepared in distilled water and was filtered in sterile conditions through a millipore membrane (pore size = 0.2 μm). The solution was directly added to the liquid culture medium at final concentrations of 1, 3, and 8 mM in the same time that inoculum. The inoculum (10^7 cells/mL) was obtained from an overnight culture of the yeasts grown in liquid culture medium. Cells in stationary phase (10^7 cells/mL) not treated with sodium orthovanadate were inoculated on Sabouraud solid medium (2% agar) plates containing sodium orthovanadate at the same concentration as that of the liquid culture medium.

The calcofluor white and Hygromycin B resistant tests were performed by adding different concentrations of calcofluor white and Hygromycin B to Sabouraud plates. Plates were inoculated with yeasts (10^7 cells/mL) at the beginning of each stationary phase grown from liquid culture medium supplemented with different concentrations (1, 3, 8 mM) of sodium orthovanadate.

Extraction of phosphopeptidomannans.—Phosphopeptidomannans were extracted as described by Peat et al.[20] with the following modifications. Whole cells were autoclaved with 0.02 mM sodium acetate buffer (pH 7). The crude extract was filtered through a membrane (pore size = 0.2 μm) and the filtrate was precipitated with ethanol for 12 h at 4°C. The pellet was diluted in distilled water, dialysed against water for 12 h and then lyophilized. The total protein content of the phosphopeptidomannans (PPM) was estimated according to the method of Lowry et al.[21] Albumin was used as a control. Samples for amino acid determination were hydrolysed for 60 min at 150°C in the presence of 6N HCl, 2% DMSO, in a Pico-Tag workstation (Waters, Milford, MA, USA) and detected at 254 nm. The total carbohydrate content was estimated by use of the phe-

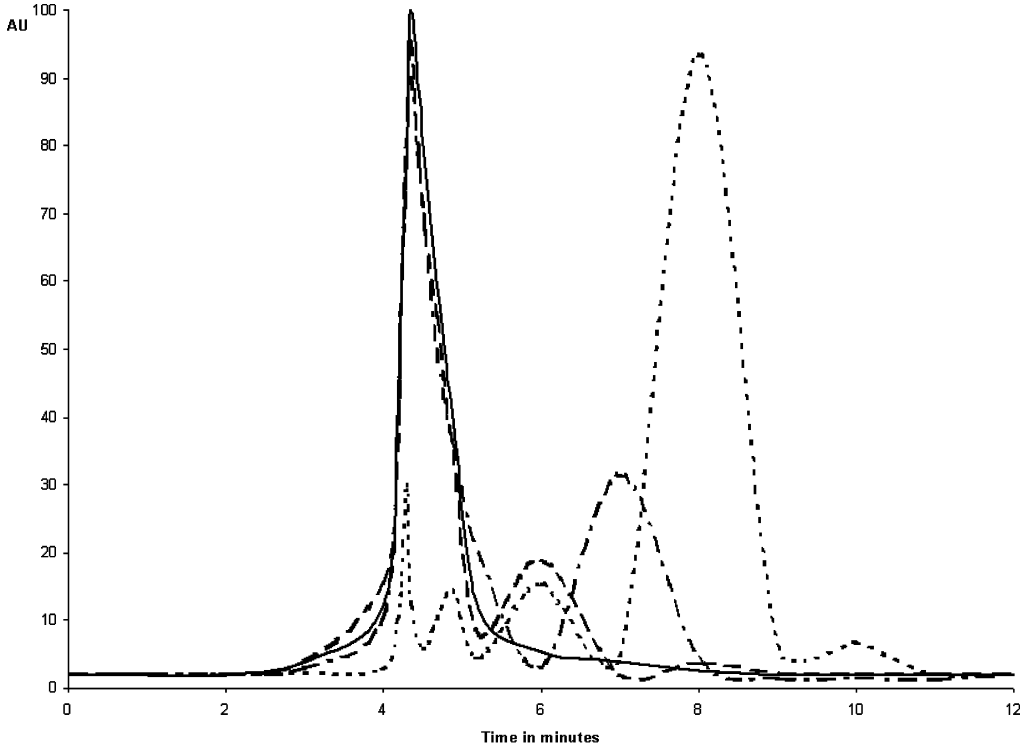

Fig. 2. Chromatogram of PPM extracted from cells and denaturated by imadazole buffer. ––– Control; –– –– –– 1 mM sodium orthovanadate; –– - –– 3 mM sodium orthovanadate; ------8 mM sodium orthovanadate.

nol/sulphuric acid reagent[22] after hydrolysis of 1 mg of sample under vacuum with 1 mL of 2N hydrochloric acid. Glucose was used as a control.

Fractionation of the phosphopeptidomannans.—All HPLC procedures were carried out at room temperature using Gilson system. Lyophilized PPM samples were taken up in water (HPLC quality). We always applied a 1 mg/cm^3 PPM solution to the chromatography columns after filtration. Samples were treated with imidazole at room temperature and the reaction was initiated by adding 50 mM imidazole buffer, pH 5.5 for 2 h. We used 20 μL of untreated PPM samples as positive controls after equilibration and elution of the column with distilled water. The masses of the PPM were estimated with the formula: Ln (MM) = 7.26–0.26 × time (in min).

Inductively coupled plasma analysis.—PPM samples were dissolved in a denaturing solution (6 M urea, 1 mM DTT) during 12 h at 37°C[23] to obtain a highest exposure of components of the phosphopeptidomannans. The degree of denaturation was estimated by measuring the UV signal of the aromatic amino acids at 284 nm with a Fujitsu spectrophotometer. Control curves of vanadium and phosphorus were achieved with the denaturing solution. To detect vanadium and phosphorus these atoms were excited and emission of vanadium was detected at 319.311 nm and phosphorus was detected at 213.618 nm with an ICP P2000 (Perkin–Elmer).

Acknowledgements

The authors thank J. Cortot (UMR CNRS 7564, LCPME, Chimie et Electrochimie Analytiques) for the inductively coupled plasma analysis.

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