The *Saccharomyces* SUN gene, *UTH1*, is Involved in Cell Wall Biogenesis

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Abstract

Deletion of the *Saccharomyces* gene, *UTH1*, a founding member of the *SUN* family of fungal genes, has pleiotropic effects. Several phenotypes of Δ*uth1* cells including their decreased levels of mitochondrial proteins, their impaired autophagic degradation of mitochondria, and their increased viability in the presence of mammalian BAX, a pro-apoptotic regulator localized to the mitochondria, have prompted others to propose that the Uth1p functions primarily at the mitochondria. In this report, we show that cells lacking *UTH1* have more robust cell walls with higher levels of β-1,3-glucan that allows them to grow in the presence of calcofluor white (CFW) or sodium dodecyl sulfate (SDS), two reagents known to perturb the yeast cell wall. Moreover, these Δ*uth1* cells are also significantly more resistant to spheroplast formation induced by zymolyase treatment than their wildtype counterparts. Surprisingly, our data suggests that several of the enhanced growth phenotypes of Δ*uth1* cells, including their resistance to BAX-mediated toxicity, arise from a strengthened cell wall. Therefore, we propose that Uth1p’s role at the cell wall and not at the mitochondria may better explain many of its effects on yeast physiology and programmed cell death.

Keywords

*UTH1*; *PKC1*; *SUN* genes; cell wall biogenesis; β-1,3-glucan; chitin

Introduction

A founding member of the *SUN* (*SIM1*, *UTH1*, and *NCA3*) family of fungal genes, *UTH1* was originally identified in a genetic screen for *S. cerevisiae* mutants that increased the stress resistance and the replicative lifespan of yeast cells (Kennedy et al., 1995; Austriaco, 1996). Mutant cells lacking *UTH1* have longer replicative lifespans (Kennedy et al., 1995; Austriaco, 1996); are capable of growing at elevated temperatures (Austriaco, 1996; Camougrand et al., 2003); are resistant to hydrogen peroxide (Austriaco, 1996; Bandara et al., 1998) and rapamycin (Camougrand et al., 2003); and are sensitive to copper (Jo et al., 2008) and to paraquat (Austriaco, 1996; Bandara et al., 1998). Finally, *Auth1* cells are also able to grow in the presence of overexpressed pro-apoptotic mammalian BAX suggesting that *UTH1* may be involved in the regulation of yeast programmed cell death (Camougrand et al., 2003).
What does Uth1p do? Several lines of evidence have prompted Camougrand and her colleagues to propose that the Uth1p protein functions primarily at the mitochondria. First, they showed that the inactivation of \textit{UTH1} lowers the levels of mitochondrial proteins including cytochrome \textit{aa3}, \textit{c}, and \textit{b}, and citrate synthase (Camougrand \textit{et al.}, 2000). Next, they localized Uth1p to the outer mitochondrial membrane and to the cell wall (Velours \textit{et al.}, 2002). Third, as we have already noted above, they observed that deleting \textit{UTH1} allows yeast cells to survive the overexpression of mammalian BAX, a pro-apoptotic protein known to act at yeast mitochondria (Camougrand \textit{et al.}, 2003). Finally, they discovered that the autophagic degradation of mitochondria is impaired in \textit{Δuth1} cells (Kissova \textit{et al.}, 2004; Kissova \textit{et al.}, 2007). In light of this data, Camougrand \textit{et al.} have suggested that Uth1p is a regulator of mitochondrial function and that this role may explain its diverse effects on yeast apoptosis and cell physiology (Camougrand \textit{et al.}, 2004).

In this paper, we report that \textit{UTH1} is involved in cell wall biogenesis: Cells lacking \textit{UTH1} have more robust cell walls that are relatively more resistant to enzymatic attack by zymolyase than their wildtype counterparts, probably because they contain higher levels of β-D-glucan. Surprisingly, our data also suggests that several of the enhanced growth phenotypes of \textit{Δuth1} cells, including their resistance to BAX-mediated toxicity, arise from a strengthened cell wall. Therefore, we propose that Uth1p’s role at the cell wall and not at the mitochondria may better explain many of its effects on yeast physiology and programmed cell death.

\section*{Materials and Methods}

\subsection*{Yeast Strains, Plasmids, and Growth Conditions}

All experiments were done with isogenic strains in the W303-1A background (\textit{MATa ade2, his3, leu2, trp1, ura3, ssd1-d2}). The \textit{Δuth1} mutant was created by disrupting the ORF with the \textit{kanR} marker using a PCR-based knock-out strategy (Brachmann \textit{et al.}, 1998) and verified both by PCR and by phenotypic analysis. To overexpress either human BAX or yeast PKC1 in our strains, we transformed either plasmid pCM189/Bax (Camougrand \textit{et al.}, 2003) or plasmid pFR22 (Roelants \textit{et al.}, 2004) into our cells and plated them on selective media. Doxycycline supplementation was used to regulate BAX expression as previously described (Camougrand \textit{et al.}, 2003). For all the experiments described in this paper, cells were cultured using standard protocols (Amberg \textit{et al.}, 2005), and transformations were accomplished using the lithium acetate method (Gietz and Schiestl, 2007). Unless noted otherwise, all drugs were purchased from SIGMA-Aldrich.

\subsection*{Spot Assays}

Cells were grown overnight in either rich or selective media at 30°C and then diluted to a final concentration (an approximate \textit{OD}_{660} value of 0.2). For each strain, a series of 10-fold dilutions was then prepared in water over a range of concentrations from $10^{-1}$ to $10^{-5}$ relative to the initial culture. Spots of 5 μl from each dilution series were then plated to the indicated media and cultured at either 30°C or 39°C for 2, 3 or 5 days, depending upon the particular plate. Plates supplemented with drugs were poured and used on the same day. All spot assays were repeated at least three times and a representative experiment is shown.

\subsection*{Spheroplast Rate Assay}

Enzyme preparation and cell wall lysis assay were based on the method described previously (Ovalle \textit{et al.}, 1998). Briefly, cells were grown overnight in rich media at 30°C, harvested and washed three times with deionized water, and resuspended to an \textit{OD}_{660} of 0.5 in TE buffer, pH 7.5 (50 mM Tris/HCl, 5 mM EDTA). Zymolyase (5 U/μl; Zymo Research, Orange, CA) was then added to the cells to a final concentration of 12 μg/ml. Cell suspensions were incubated...
at 23°C and their optical density was recorded at four-minute intervals for the indicated time period.

**Analysis of Cell Wall Sugar Composition**

Chitin levels were quantified according to a method described previously (Lesage *et al.*, 2005). Yeast cultures were first grown to stationary phase in liquid YPD medium then diluted 1:100 in fresh YPD and incubated at 30°C with shaking for 18-22 h. Typically, one ml of culture was spun in a microfuge tube at 14K for 2 min and the media removed. The cell pellets were then air dried at 37°C for 2-3 days. Next, cell pellets were resuspended in 1 ml 6% KOH and heated at 80°C for 90 min with occasional mixing. Alkaline insoluble material was pelleted (20,000 x g, 20 min) and neutralized with phosphate-buffered saline for 10–20 min with occasional mixing. After centrifugation (20,000 x g, 20 min), 200 μl of McIlvaine’s Buffer (0.2 M Na2HPO4/0.1 M citric acid, pH 6.0) was added to pellets. Extracts were then stored at -20°C until processed for chitin measurements. Samples were thawed and subjected to digestion with 5 μl of 5 mg/ml chitinase from *Trichoderma viride* (SIGMA-Aldrich) at 37°C for 36–40 h and then for 20–24 h. The amounts of chitin were then determined by using a modified Morgan-Elson procedure as described previously (Bulik *et al.*, 2003). The levels of chitin, expressed as GlcNAc concentration, were then normalized to the dry weight of the sample.

Next, β-D-glucan levels were quantified according to the methods of Boone *et al.* (1990) and Yiannikouris *et al.* (2004). Yeast cells were grown as 10 ml cultures of YPD until stationary phase. Cells were harvested, washed once with distilled water, and then extracted three times with 0.5 ml of 3% NaOH at 75 °C (1 h per extraction). After alkali extraction, the alkali-insoluble material was washed once with 1 ml of 100 mM Tris-HCl (pH 7.5), and once with 1 ml of 10 mM Tris-HCl (pH 7.5). The washed residue was then digested for 16 h at 37 °C with 1 mg of Zymolyase 100T (United States Biological, Swampscott, MA), in 1 ml of 10 mM Tris-HCl (pH 7.5). The insoluble pellet that remains after zymolyase digestion was removed by centrifugation, and the supernatant was dialyzed against distilled water, using Spectra/por tubing with a 6,000-8,000-D pore size (Spectrum Laboratories, Rancho Dominguez, CA), for 16 h. Carbohydrate content prior to dialysis [(1, 3) plus (1, 6) β-glucan] and post dialysis [(1, 6) β-glucan alone] was measured as hexose by the phenol-sulfuric acid method (Dubois *et al.*, 1956). The levels of β-D-glucan, were then normalized to the dry weight of the sample. Finally, supernatants obtained from the alkali extraction containing alkali soluble β-glucans and mannoproteins from yeast cell walls were dialyzed (1:100, v/v) with 0.02 M Tris-HCl buffer (pH 7.4) for at least 16 h at 4 °C with 0.02 M Tris-HCl buffer (pH 7.4). Mannans and β-glucans were separated on a concanavalin A Sepharose column (Pharmacia) at 4°C as previously described (Yiannikouris *et al.*, 2004). Alkali-soluble β-glucans were eluted with 0.02 M Tris-HCl buffer (pH 7.4)/0.5 M NaCl and stored at -20 °C until carbohydrate content was measured as above, using the phenol-sulfuric acid method.

**Results and Discussion**

Deleting *UTH1* has pleiotropic effects including phenotypes associated with the endoplasmic reticulum

Deletion of the gene, *UTH1*, has pleiotropic effects. As we and others have previously shown, mutant cells lacking *UTH1* are capable of growing at elevated temperatures; are resistant to hydrogen peroxide; and are sensitive to copper and to paraquat (Figure 1). In addition, we have also discovered that they are resistant to β-mercaptoethanol (Figure 1) and to dithiothreitol (data not shown), drugs known to induce ER stress (Cox and Walter, 1996). All of these phenotypes suggest that *UTH1* function involves numerous physiological processes in the cell.
**UTH1 is involved in maintaining the integrity of the yeast cell wall**

UTH1 is a founding member of the SUN family of fungal genes (Austriaco, 1996). Four other fungal SUN genes, SUN4 in *S. cerevisiae* (Mouassite et al., 2000), *psu1* in *S. pombe* (Omi et al., 2005), and SUN41 and SIM1/SUN42 in *C. albicans* (Hiller et al., 2007; Firon et al., 2007; Sosinska et al., 2008), have been implicated in the regulation of the integrity of the yeast cell wall. To test if UTH1 has a similar function, we plated wildtype and Δuth1 cells on media containing either calcofluor white (CFW) or sodium dodecyl sulfate (SDS), two reagents known to perturb the yeast cell wall (Kaeberlein and Guarente, 2002), and observed that the mutant is more resistant to these agents than the wildtype strain (Figure 2A). This suggested that deleting UTH1 strengthens the yeast cell wall.

To further characterize this phenotype, we plated wildtype and Δuth1 cells on CFW and on SDS plates supplemented with 1 M sorbitol, which provides osmotic stabilization and prevents lysis caused by a weakened cell wall (Kaeberlein and Guarente, 2002). Under these conditions, there is no difference in the growth of our wildtype and mutant strains suggesting that the deletion of UTH1 phenocopies the supplementation of growth media with an osmotic stabilizer (Figure 2A).

To confirm our findings, we overexpressed *PKC1* in wildtype and Δuth1 cells using a high copy plasmid and plated them on SDS plates with or without osmotic stabilization. Pkc1p is a central integrator of cell integrity that acts to promote transcription of cell wall biosynthetic genes (Heinisch et al., 1999). We observed that the growth rates of wildtype cells, wildtype cells overexpressing *PKC1*, and Δuth1 cells, suggesting that deleting UTH1 and overexpressing *PKC1* have the same effect of enhancing cell wall integrity (Figure 2B). Since we see no difference in growth between wildtype cells overexpressing *PKC1*, wildtype cells on sorbitol, and wildtype cells overexpressing *PKC1* on sorbitol, our data suggests that the phenotypes we observe from either of these manipulations – either the overexpression of *PKC1* or the supplementation of media with 1 M sorbitol – can be attributed directly to their enhancement of cell wall integrity rather than to an indirect effect caused either by a non-cell wall-related function of overexpressed *PKC1* (Fairn et al., 2007) or by the sorbitol-mediated activation of the osmoregulatory HOG pathway (Saito and Tatebayashi, 2004). Otherwise, we would have seen a synergistic effect on the growth of wildtype cells overexpressing *PKC1* on SDS plates supplemented with sorbitol.

Finally, to more directly assay the structural integrity of the wildtype and Δuth1 yeast cell walls, we compared the rates of formation of spheroplasts of wildtype and mutant Δuth1 yeast cells cultured in a hypotonic solution in the presence of zymolyase, a mixture of cell wall-digesting enzymes (Ovalle et al., 1998). This spheroplast rate assay has been used by others to show that Acb1p (Gaigg et al., 2001), Pho85p (Huang et al., 2002), and Bet1p (Kipnis et al., 2004) are all involved in maintaining the integrity of the yeast cell wall. As shown in Figure 3, mutant cells lacking UTH1 were significantly more resistant to zymolyase treatment than wildtype cells. More specifically, for wildtype cells, the mean lag time (LT) for three independent samples – where lag time (LT) has been estimated by interpolation of the lysis curve as the time in which the OD_{660} dropped by 0.05 from its initial value (Ovalle et al., 1998) – was 12 min and the mean maximal lysis rate (MLR) was 0.230 – where MLR has been defined as the absolute value of the slope of the least-squares fit line for the portion of the lysis curve with the steepest log-linear decline (Ovalle et al., 1998). In contrast, for the Δuth1 mutants, the mean lag time (LT) for three independent samples was 18.6 min and the MLR was 0.074. The rate indices (RI) of the wildtype and Δuth1 cells, where RI has been defined as MLR/LT (Ovalle et al., 1998), were 0.0192 and 0.0041 respectively (p<0.002). These results demonstrate that the walls of Δuth1 cells are more resistant to enzymatic attack.
Deleting *UTH1* alters the polysaccharide composition of the yeast cell wall

How does *UTH1* regulate the integrity of the yeast cell wall? Firon et al. have shown that *UTH1* is able to complement a *sun41Δ sun42Δ* double mutant in *C. albicans* (Firon et al., 2007). Given that *SUN41* and *SIM1/SUN42* mutants manifest specific cell wall defects at the septa in *Candida* and that these *Candida* mutants are sensitive only to cell wall-perturbing agents that are specific to chitin synthesis, these authors have proposed that *UTH1* is involved in chitin biosynthesis in *S. cerevisiae*. To test this hypothesis, we determined the chitin levels in the cell walls of *Auh1* cells and showed that they indeed have decreased amounts of chitin as compared to wildtype controls (Table 1).

This finding – that *Auh1* cells have lower chitin levels – was unexpected. As shown in Figure 2 and Figure 3, *Saccharomyces* cells lacking *UTH1* are not only more resistant to calcofluor white (CFW) and to sodium dodecyl sulfate (SDS), two reagents that are known to destabilize the cell wall (Kaeberlein and Guarente, 2002), but are also significantly more resistant to zymolyase, a mixture of cell wall-digesting enzymes (Kitamura and Yamamoto, 1972). This would not be expected if deleting *UTH1* only lowers the chitin content of the cell wall, an effect expected to weaken and not to strengthen the cell wall. Interestingly, one previous report has shown that mutant cells with increased resistance to CFW have lower chitin levels than their wildtype counterparts (Roncero et al., 1988).

How could lower levels of chitin lead to the strengthening of the yeast cell wall? Some have suggested that a compensatory mechanism exists in yeast in response to cell wall damage whereby decreases in β-1,3-glucan levels leads to a compensatory increase in chitin levels (Kapteyn et al., 1997; Popolo et al., 1997; Ram et al., 1998; Valdivieso et al., 2000). To explain our data, therefore, we predicted that the reverse mechanism may also exist: The lower levels of chitin in *Auh1* cells may be accompanied by compensatory higher levels of β-1,3-glucan. To test this hypothesis, we determined the β-1,3-glucan levels of *Auh1* cells. Indeed, as predicted, these mutant *Auh1* cells had cell walls with significantly higher levels both of total alkaline soluble and total alkaline insoluble β-1,3-glucan and of alkaline insoluble (1,6) β-1,3-glucan more specifically (Table 1). This would explain why *Auh1* cells are more resistant to zymolyase, a mixture of cell wall-digesting enzymes composed primarily of β-1,3-glucan laminaripentaohydrolase and β-1,3-glucanase (Kitamura and Yamamoto, 1972). The higher levels of β-1,3-glucan would also explain our genetic data that had suggested that the cell walls of *Auh1* cells are more robust than their wildtype counterparts.

In sum, our data suggests that *UTH1* is involved in the biogenesis of the yeast cell wall. The precise molecular mechanism behind this role, however, remains unknown: Uth1p could be involved in any of the regulatory pathways that have been linked to the complex process of cell wall assembly in *Saccharomyces cerevisiae* (Lesage and Bussey, 2006). It is significant that the Uth1p homolog in *Candida albicans*, Sim1p, has been identified as a covalently linked cell wall protein by mass spectrometry (Sosinska et al., 2008). Another group has also shown that Uth1p appears to act in concert with another SUN protein, Sun4p, to contribute to cell wall morphogenesis and septation strongly supporting our proposal that the protein is involved in
Deleting UTH1 improves growth under diverse stress conditions by enhancing cell wall integrity

Finally, to determine if UTH1’s role in regulating the integrity of the yeast cell wall could explain any of the pleiotropic phenotypes of the Δuth1 mutant, we took wildtype and Δuth1 cells and stressed them on media with or without sorbitol supplementation. We discovered that there is no difference in growth between these two strains either at 39°C, on media containing either β-mercaptoethanol or copper, or with overexpressed mammalian BAX, as long as they are cultured on media with an osmotic stabilizer (Figure 4A). This suggested that the growth enhancement attributed to the deletion of UTH1 under these conditions could be explained by the gene’s role in cell wall biogenesis rather than on mitochondrial function. This was true regardless of whether the BAX cell death assays were done on sorbitol-supplemented media containing either glucose or glycerol as a carbon source (data not shown). In both cases, the viability of wildtype and Δuth1 cells overexpressing mammalian BAX were indistinguishable.

To confirm this result, we repeated the assay with wildtype and Δuth1 cells both overexpressing PKC1 on a high copy plasmid and observed that the growth rates of wildtype cells, wildtype cells overexpressing PKC1, and Δuth1 cells, at 38°C are indistinguishable on plates supplemented with sorbitol, suggesting once again that the enhanced growth phenotype of Δuth1 cells at elevated temperatures is linked to the enhancement of cell wall integrity (Figure 4B). As before, the absence of a synergistic effect on the growth at 38°C of wildtype cells overexpressing PKC1 on plates supplemented with sorbitol suggested that the growth enhancement phenotype could be attributed directly to a strengthened cell wall. Finally, parallel results were obtained on copper supplemented plates suggesting that the sensitivity of Δuth1 cells to copper is also mediated by the gene’s effect on the integrity of the cell wall (data not shown). It would be interesting to determine if any of the other phenotypes of Δuth1 cells, especially those associated with Uth1p’s putative mitochondrial function, can also be linked to its role in regulating the integrity of the yeast cell wall. Finally, in light of our findings it is intriguing to note that one group has been unable to find the reported link between Uth1p function and mitochondrial autophagy (Kanki et al., 2009). However, it is still not clear if this was due to differences in strain background. If so, we speculate that Uth1p’s differential effects on mitophagy could be linked to the differences in cell wall composition commonly seen among wildtype yeast strains.

In conclusion, we show that cells lacking UTH1 have more robust cell walls that are resistant to zymolyase treatment because they contain higher levels of β-1,3-glucan. Surprisingly, our data also suggests that several of the enhanced growth phenotypes of Δuth1 cells, including their resistance to BAX-mediated toxicity, arise from a strengthened cell wall. Thus, we propose that Uth1p’s role at the cell wall and not at the mitochondria may better explain many of its effects on yeast physiology and programmed cell death.

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*Saccharomyces cerevisiae* Cell Wall and Study of Their Adsorptive Properties toward Zearalenone.
Figure 1.

UTH1 is involved in the yeast cell’s response to different stresses. 5 μl aliquots of 10-fold serial dilutions of wildtype and Δuth1 mutant cells in the W303-1A strain background were plated onto the designated media and cultured at either 30°C or 39°C for 2, 3 or 5 days, depending upon the particular plate. All spot assays were repeated at least three times and a representative experiment is shown.
Figure 2.

**UTH1** is involved in maintaining the integrity of the yeast cell wall. (A) Deleting **UTH1** enhances cell growth on media supplemented with calcofluor white (CFW) or sodium dodecyl sulfate (SDS). 5 μl aliquots of 10-fold serial dilutions of wildtype and Δuth1 mutant cells in the W303-1A strain background were plated onto the designated media and cultured at 30°C for 2 or 4 days, depending upon the particular plate. YPDS denotes YPD plates supplemented with 1 M sorbitol respectively. (B) Overexpression of **PKC1** enhances cell wall integrity mimicking a deletion of **UTH1**. 5 μl aliquots of 10-fold serial dilutions of wildtype and Δuth1 mutant cells transformed with plasmid pFR22 to overexpress **PKC1** were plated onto the designated selective SD plates and cultured at 30°C for 2 or 5 days. SD-S denotes SD plates supplemented with 1 M sorbitol. All spot assays were repeated at least three times and a representative experiment is shown.
Figure 3.
Deleting *UTH1* decreases the rate of spheroplast formation of yeast cells. Cells of the indicated genotypes were cultured overnight in rich YPD media at 30°C, harvested and washed three times with deionized water, and then resuspended to an OD$_{660}$ of 0.5 in TE buffer, pH 7.5 (50 mM Tris/HCl, 5 mM EDTA). Zymolyase (5 U/μl; Zymo Research, Orange, CA) was then added to the cells to a final concentration of 12 μg/ml. Cell suspensions were incubated at 23°C and their optical density (OD$_{660}$) was recorded at four-minute intervals for the indicated time period. Assays were done in triplicate and mean values are shown along with the standard deviation.
Deleting *UTH1* enhances the cell’s response to diverse stresses by strengthening its cell wall. (A) Osmotic stabilization with 1M sorbitol phenocopies a deletion of *UTH1*. 5 μl aliquots of 10-fold serial dilutions of wildtype and Δ*uth1* mutant cells were plated onto the designated media and cultured at either 30°C or 39°C for 2, 3 or 5 days. YPDS and SD-S denotes YPD and SD plates supplemented with 1 M sorbitol respectively. To overexpress human BAX in our strains, we transformed plasmid pCM189/Bax into our cells and plated them on selective SD media with or without doxycycline supplementation as previously described (Camougrand et al., 2003). (B) Overexpression of *PKC1* enhances cell growth at elevated temperatures mimicking a deletion of *UTH1*. 5 μl aliquots of 10-fold serial dilutions of wildtype and Δ*uth1* mutant cells transformed with plasmid pFR22 to overexpress *PKC1* were plated onto the designated selective SD plates and cultured at 30°C or 38°C for 2 or 5 days. All spot assays were repeated at least three times and a representative experiment is shown.

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Table 1

Chitin and Glucan Composition of the Cell Wall

Deleting *UTH1* alters the polysaccharide content of the yeast cell wall. Chitin and β-D-glucan levels were quantified as described in Materials and Methods. Assays were done in triplicate and mean values are shown along with the standard deviation in brackets. Statistical significance was calculated with the Student’s unpaired t-test.

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<th>Chitin</th>
<th>GLUCAN</th>
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<tr>
<td></td>
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<td>Alkaline Soluble</td>
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<tr>
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<td>1,6 β–glucan (μg/g dry weight)</td>
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<td>90 (22)</td>
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<td>Total β–glucan (μg/g dry weight)</td>
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<td>247 (109)</td>
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