Characterization of α,α-trehalase released in the intestinal lumen by the probiotic Saccharomyces boulardii

JEAN-PAUL BUTS1, CATHARINE STILMANT1, PAUL BERNASCONI2, CHRISTIANE NEIRINCK1 & NADINE DE KEYSER1

1Faculty of Medicine, Université Catholique de Louvain, Laboratory of Paediatric Gastroenterology and Nutrition, Cliniques St Luc, Brussels, Belgium, and 2Laboratoires Biocodes, Gentilly, France

Abstract

Objective. Trehalose intolerance due to α,α-trehalase deficiency has scarcely been studied. The purpose of this study was to measure α,α-trehalase activity in intestinal biopsy samples from 200 consecutive patients over a period of 6 months, and to characterize α,α-trehalase released by the probiotic Saccharomyces boulardii (S. boulardii). Material and methods. Enzyme activities were measured in human and rat intestinal mucosal samples using the micromethod of Messer & Dalqvist. α,α-Trehalase from S. boulardii was immunoprecipitated and Western blotted using an IgG purified antibody raised against a 23 amino acid peptide of α,α-trehalase of S. cerevisiae. Results. Among 200 patients, most of whom complained of abdominal symptoms and diarrhoea, 18 (9%) had total α,α-trehalase deficiency (0-12 U/g mucosa) and 39 had partial deficiency (3-12 U/g mucosa). Only 4 patients (2%) presented selective α,α-trehalase deficiency with otherwise normal disaccharidases. Expressed per gram of powder, α,α-trehalase from S. boulardii delivered in vitro an activity 175 times higher than that of human trehalase per gram of intestinal mucosa. Vmax (22 ± 0.43 μmol) and Km (5 mM) were close to that of the human enzyme, whereas Western blot revealed a signal of two subunits of 82 kDa. Finally, treatment of rats with S. boulardii resulted in increases in α,α-trehalase activities of 25 to 45% (p < 0.01) in endoluminal fluid and intestinal mucosa compared with controls. Conclusions. Our data suggest that α,α-trehalase deficiency is more common than is believed and that oral administration of S. boulardii could be beneficial in patients with digestive symptoms caused by trehalase intolerance.

Key Words: α,α-Trehalase, human small intestine, probiotic, rat small intestine, Saccharomyces boulardii

Introduction

Trehalase is a disaccharide, the main dietary source of which is mushrooms. It has been approved as an additive in the preparation of dried foods. Isolated intestinal trehalase deficiency is found in 8% of Greenlanders [1]. Although congenital trehalase deficiency is rare [1], secondary trehalase deficiency is more usual. Activity may be significantly reduced in untreated coeliac disease, in acute and chronic enteropathies and in patients with chronic abdominal symptoms of unknown origin. Arola et al. [2,3] showed that malabsorption of trehalase causes abdominal symptoms similar to those in lactase intolerance including flatus, hypermetabolism and loose stools or diarrhoea. Among 64 adult subjects with abdominal symptoms, 19 experienced clear signs of trehalose intolerance in response to a 25-g oral trehalose load test. Murray et al. [4] estimated that 2% of the Caucasian population has a very low activity of trehalase (below 4 U/g protein) or has virtually no enzyme activity at all. Like sucraser-isomaltase, human α,α-trehalase is anchored to the intestinal microvillus membrane, and is a homodimeric enzyme and a glycoprotein with glycosidase activity.

Saccharomyces boulardii is a non-pathogenic yeast exhibiting therapeutic properties in acute and chronic enteropathies, irritable bowel syndrome, antibiotic-associated diarrhoea, and enterotoxigenic Clostridium difficile infections [5,6]. In human volunteers [7] and in growing rats [8], lyophilized preparations of S. boulardii produce trophic intestinal effects including increases in the specific and

Correspondence: Jean-Paul Buts, MD PhD, Faculty of Medicine, Université Catholique de Louvain, Laboratory of Paediatric Gastroenterology and Nutrition, Unit of Paediatrics, 10 Avenue Mounier, Cliniques St Luc, Brussels, BE-1200 Belgium. E-mail: baps@upmc.ulg.ac.be

(Received 8 April 2008; accepted 16 June 2008)

ISSN 0036-5521 print ISSN 1502-7708 online © 2008 Informa UK Ltd.
DOI: 10.1080/00365520802308862
total activities of brush-border membrane enzymes [7,8], enhanced secretion of s-IgA in intestinal fluid [9] and marked stimulation of sodium-dependent, D-glucose uptake with a corresponding increase in the sodium D-glucose co-transporter-1 (SGLT-1) [8]. These effects are at least partly mediated by the endoluminal release of polyamines [10], as yeast cells contain substantial amounts of spermine and spermidine [11], and by the endoluminal release of yeast enzymes such as sucrase [7,12], leucine aminopeptidase [13] and a novel protein phosphatase that inhibits Escherichia coli endotoxin by dephosphorylation [14,15].

To further study the trophic effects of the probiotic S. boulardii, we identified and characterized the properties (pH optimum, \(K_m, V_{max}\), mol weight) of a yeast, \(\alpha,\alpha\)-trehalase, isolated from S. boulardii cells, and measured its activity in the lumen and intestinal mucosa of rats treated with S. boulardii or with saline controls. We also measured the enzyme activity in the lyophilized preparation of S. boulardii and in intestinal biopsy samples from 200 consecutive patients along with other disaccharidase activities.

Material and methods

Media and culture conditions

S. boulardii cells were inoculated in YPD (yeast extract, 0.5%; peptone, 2%; glucose, 2%; DIFCO, Detroit, Mich., USA) media and were grown at 30°C with moderate shaking, as described [13].

To disrupt the external capsid, yeast cells were concentrated (1.45–1.50 × 10^{10} cells/ml) and shaken with beads (diameter 0.45–0.50 mm) under cold CO_2 flux by using an MSK pulse device (Braun, Paris, France) [13]. After stabilization in phosphate-buffer 0.01 M (pH 7.0), or maleate buffer 0.1 M (pH 6.5), particulate components were removed by centrifugation (5000 g) for 15 min at 0°C and the supernatants were stored at −70°C in liquid nitrogen until analysed.

Animals and treatments

The study was approved by the Animal Welfare Committee and the Human Ethics Committee of the Catholic University of Louvain, Faculty of Medicine. Litters of rats were reduced to six pups per lactating mother to equalize conditions of nursing and feeding. For ontogenic studies, pups were killed during the suckling, weaning and post-weaning periods. To determine whether, oral administration of lyophilized S. boulardii can influence endoluminal \(\alpha,\alpha\)-trehalase activity, growing rats were treated from day 15 to day 20 postpartum because at this time, \(\alpha,\alpha\)-trehalase activity is low in the rat jejunum as well as in the ileum.

S. boulardii was prepared in lyophilized form (100 mg per flask, biologic activity 2.9 × 10^9 viable cells/ml) by the manufacturer (Biocodex, Gentilly, France). As previously reported [7–10], we used a dose of 50 μg lyophilized yeast cells per gram body weight (b.w.) per day. The appropriate dose was administered in 0.1 ml saline by nasogastric intubation twice daily from day 15 to day 20. Control groups were treated according to the same schedule and received equal volumes of saline. Nine to 11 animals per group were studied during the weaning period of induction of the enzyme activity.

Collection of endoluminal fluid

On day 20 postpartum, the rats were killed by decapitation, and the small intestine from the pylorus to the ileocaecal valve was immediately excised. The total length was measured and divided into two equal segments. The proximal half was considered the jejunum and the distal half the ileum. For collection of intestinal endoluminal fluid, jejunal and ileal segments were flushed with 2 ml cold 0.9% saline. The collected fluid was centrifuged (500g, 5 min) and the supernatants were pooled and filtered through a 0.2-μm membrane filter (Millipore Corp., Bedford, Mass., USA) to discard yeast cells in suspension. As a result, the enzyme activity measured was the total endoluminal enzyme activity delivered both by oral S. boulardii and by intestinal cells extruded from the villi.

Intestinal mucosal biopsy samples

\(\alpha,\alpha\)-Trehalase activity was also assayed on homogenates of duodenal+junal biopsy samples from 200 children and adult patients who underwent an upper gastrointestinal (GI) tract endoscopy for digestive symptoms. Activity of the other disaccharidases (lactase, maltase and saccharase-isomaltase) was also assayed.

Lyophilized preparations of S. boulardii

\(\alpha,\alpha\)-Trehalase activity was also measured on lyophilized preparations of S. boulardii, freshly prepared by the manufacturer (Biocodex) on cells having a biological activity of 2.9 × 10^9 viable cells.

Enzyme assays

\(\alpha,\alpha\)-Trehalase activity was measured on suspensions of S. boulardii cells, intestinal fluid, purification buffers and intestinal mucosal samples using trehalase as
Table 1. Among 20 species of microorganisms (bacteria, fungi) matching with the sequence chosen of 23 amino acids (DPFRVGEYNGQADFKGAATEG)⁶ σ,α-trehalase of *Saccharomyces cerevisiae* on Blast search, 64⁶ 700 the most representative matching sequences are listed below.

<table>
<thead>
<tr>
<th>Enzyme (EC 3.2.1-28)</th>
<th>Species</th>
<th>Identities</th>
<th>Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. σ,α-trehalase neutral</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>23/23</td>
<td>100%</td>
</tr>
<tr>
<td>2. σ,α-trehalase neutral</td>
<td><em>Enterobacter aerogenes</em></td>
<td>21/23</td>
<td>91%</td>
</tr>
<tr>
<td>3. σ,α-trehalase neutral</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>21/23</td>
<td>91%</td>
</tr>
<tr>
<td>4. σ,α-trehalase neutral</td>
<td><em>Candida glabrata strain CR9138</em></td>
<td>21/23</td>
<td>91%</td>
</tr>
<tr>
<td>5. σ,α-trehalase neutral</td>
<td><em>Candida albicans strain SC5314</em></td>
<td>19/22</td>
<td>86%</td>
</tr>
<tr>
<td>6. σ,α-trehalase neutral</td>
<td><em>Arthrosporea nidulans</em></td>
<td>19/23</td>
<td>82%</td>
</tr>
<tr>
<td>7. σ,α-trehalase neutral</td>
<td><em>Schizosaccharomyces pombe</em> (fission yeast)</td>
<td>18/23</td>
<td>78%</td>
</tr>
</tbody>
</table>

substrate in maleate buffer (0.56 M, pH 6.0) using the micromethod of Messer & Dahlqvist [16].

Unless otherwise indicated, assays were performed at 37°C for 60 min. One unit equals 1 μmol of glucose formed per minute and per gram of protein. Protein content was determined by the method used by Lowry et al. [17]. By convention, total enzyme activity was considered for activities ranging from 0 to 2 U/g protein⁻¹, while activities ranging from 3 to 12 U/g protein⁻¹ were considered as a partial deficiency.

Immunoprecipitation and immunoblotting

To demonstrate the production by *S. boulardii* of an enzyme protein with σ,α-trehalase activity, a peptide (DPFRVGEYNGQADFKGAATEG)⁶ corresponding to a highly conserved sequence of σ,α-trehalase of *S. cerevisiae* was synthesized (see Table).

This peptide matched with σ,α-trehalase of 20 species of microorganisms (bacteria and fungi) including *Schizosaccharomyces pombe* (78% identities with the above sequence). The Table presents a list of the most representative microorganisms (match 78–100%) whose σ,α-trehalase has identities in common (18/23 to 23/23) with the sequence synthesized. Rabbits were immunized with the above peptide corresponding to a 23 amino acid sequence of *S. cerevisiae*. A polyclonal antiserum was generated and IgG were purified. Fresh samples of viable *S. boulardii* were suspended in phosphate buffer (0.01 M, pH 7) containing antiproteases (leupeptine, pepstatin, PMSF). The samples were ultracentrifuged at 102,000g at 4°C for 80 min. The supernates were collected and again centrifuged 20,000g at 4°C for 25 min. The collected supernates were frozen in liquid nitrogen at -170°C until use. Protein concentrations were measured in samples of the supernates (~47 mg/mL).

On the day of the experiment, *S. boulardii* supernates were diluted, respectively, 4-, 10-, 16- and 40-fold with radioimmunoprecipitation assay (RIPA) buffer (TRIS 0.025 M, Trition X100 0.5%, Non-Ide P40 0.5%) containing antiproteases (10 μl) pepstatin, leupeptin and PMSF. To *S. boulardii* suspensions, 100 μl protein A sepharose 4B, diluted in RIPA buffer (1/1), was added to each sample and mixed by rotation for 4 h at 4°C. Thereafter, the samples were centrifuged at 24,000g for 5 min at 4°C. The pre-cleared supernates were collected and mixed with 10 μl IgG purified anti-σ,α-trehalase antibodies by rotation overnight at 4°C. Thereafter, 100 μl protein A sepharose 4B was added to the samples and mixed by rotation for 4 h at 4°C. The sepharose beads were then washed twice with RIPA buffer and once with TRIS 10 mM buffer. After the last wash, 5 μl bromophenol blue (Invitrogen, Carlsbad, Calif., USA) diluted in 10 μl aqua milliQ was added to the beads, the supernates having been discarded. Immunoprecipitation and immunoblotting were carried out using the one-step complete IP-Western kit (Genescript Corporation, Piscataway, N.J., USA). This novel procedure allows the detection of nanograms of antigen by chemoluminescence (ECL) without showing co-immunoprecipitation of the heavy and light chains of the IgG antibody.

![Figure 1. Correlation between trehalase activity (n=74) and lactase activity (n=74) in patients with low lactase activity. The correlation (r=0.56) is positive and is highly significant. (p <0.0001).](image-url)
Calculations and statistics

Statistical analyses were carried out using ANOVA followed by a multiple comparison test (Newman-Keuls) using GraphPad Prism4.0 (GraphPad Software, San Diego, Calif., USA). Differences between means were considered to be statically significant when the p-value was less than 0.05. All units represent means ± SD, unless otherwise indicated.

Results

Patients

From October 2006 to April 2007, biopsy samples collected from the distal duodenum (3rd and 4th segments) of 144 adults (mean age 43 ± 17 years) and 56 children (mean age 5 ± 2 years) were analysed specifically for the activity of disaccharidases including the activities of α,β-trehalase, neutral lactase-phlorizin hydrolase, sucrase-isomaltase and maltase-glucosaminidase. Adults underwent biopsies mainly for lactase deficiency and abdominal symp-
toms due to lactose intolerance, irritable bowel syndrome and digestive complaints of unknown aetiology including abdominal distension, cramps and diarrhoea. Children underwent biopsies mainly because of suspected coeliac disease, food intolerance, lactase deficiency and chronic enteropa-thies with diarrhoea. Among the 200 biopsy samples (105 F, 95 M), 18 patients (9%) had total deficiency in α,β-trehalase (0–2 U/g protein), 39 (19.5%) had partial deficiency (3–12 U/g protein) while the remainder of the group (145) had normal α,β-trehalase activity (13–96 U/g protein) (71.5%). Thus, 57 patients (30 adults and 27 children) had total or partial trehalase deficiency which in this series represents 28.5%.

Considering patients with α,β-trehalase deficiency with otherwise normal disaccharidases activities, 4 patients on 200 (2%) were found to have an isolated total α,β-trehalase deficiency (0–2 U/g protein) while 14 patients among 200 (7%) had alactasia (0–2 U/g) with an otherwise normal enzyme pattern. In Figure 1 it is shown that, for

![Diagram](image)

Figure 2. Variations in α,β-trehalase activity measured on suspensions of *S. boulardii* cells in relationship with variations of the pH buffer. The enzyme activity peaked between pH4 and pH 6 and required the presence of cofactors, since addition of EDTA (20mM) significantly lowered the enzyme activity.
the whole group, there was a significant ($p < 0.001$ $R = 0.56$) correlation between the activity of $\alpha\alpha$-trehalase and that of neutral lactase, which suggests that $\alpha\alpha$-trehalase is readily denatured, like neutral lactase.

However, for 74 patients with partial or total, $\alpha\alpha$-trehalase and/or neutral lactase deficiency, the ratio of trehalase/lactase was $2.56 \pm 5.69$ (SD) with a range of 0 to 37.7, while for the same patients the ratio of trehalase/sucrase was $0.40 \pm 0.16$ (SD) with a range of 0 to 1.13, indicating that the ratio of trehalase/sucrase is a much better parameter in confirming $\alpha\alpha$-trehalase deficiency because of much closer values than the ratio trehalase/lactase, which has a very wide scatter of values.

$\alpha\alpha$-trehalase released from S. bouardii

$\alpha\alpha$-trehalase activity and protein content were first measured in suspensions of S. bouardii viable cells. S. bouardii supplied about 700 units per gram of lyophilized powder, whereas the human small intestine exhibited an activity of only 4 units per gram of mucosa. Figure 2 shows variations in $\alpha\alpha$-trehalase activity measured on suspensions of S. bouardii cells in relationship to variations of the pH buffer. The enzyme activity peaked between pH 4 and 6, and required the presence of co-factors ($Ca^{2+}$, $K^+$) since the addition of EDTA (20 mM) significantly lowered the enzyme activity.

Because we used a homogenate of whole cells, it is likely that the enzyme activity measured reflected both neutral soluble and acid vacuolar $\alpha\alpha$-trehalase [18,19], with a resulting pH of 5. As shown in Figure 3, $V_{max}$ was $22 \mu$mol $\pm 0.43$, while the $K_m$ was estimated to be 5 mM. These values are close to those measured for $\alpha\alpha$-trehalase of S. cerevisiae [18]. Figure 4 shows an immunoblot with 4 lines (A, B, C, D) of immunoprecipitated $\alpha\alpha$-trehalase either with the whole antisera (A, B, C) resulting in a weak signal or with the purified IgG fraction (D) resulting in a stronger signal of two subunits of 80-82 kDa.

This mol mass is close to the molecular mass estimated for the neutral $\alpha\alpha$-trehalase monomer of S. cerevisiae (86 kDa) [19,20].

Animal treatment

Ontogenic changes of $\alpha\alpha$-trehalase activity in the jejunum and ileum of growing rats (Figure 5) revealed that the enzyme activity is virtually absent during the suckling period, as is sucrase [11], and is triggered mainly in the jejunum by day 16 postpartum. In consequence, we treated weaning growing rats with lyophilized S. bouardii (50 mg/kg b.w.) given by nasogastric intubation of 0.1 ml twice daily from day 15 to day 20. Control rats were treated with equivalent volumes of saline 0.9%, twice
daily during the same period. We found no difference between the two groups in body-weight gain, intestinal length, mucosal mass and intestinal protein content. The results of \( \alpha,\alpha \)-trehalase activity measured in the endoluminal fluid and in the intestinal mucosa, expressed in milligrams per milliliter of protein and in milligrams per millilitre of endoluminal fluid, are depicted in Figure 6. In the endoluminal fluid, filtered to discard \( S. boulardii \) cells, the enzyme activity was significantly increased by 25% and 40% compared with the activity measured in control rats, while in the intestinal mucosa, the activity was at least two times higher in treated rats than in controls.

**Discussion**

When given orally as a lyophilized preparation, \( S. boulardii \) supplies a high level of \( \alpha,\alpha \)-trehalase activity in the small-intestinal lumen that represents an activity 157 times higher than that of \( \alpha,\alpha \)-trehalase measured in the human small intestine, when data are expressed per gram of powder and per gram of intestinal mucosa. This massive supply in \( \alpha,\alpha \)-trehalase by \( S. boulardii \) is further confirmed by the significant increases in enzyme activity in the intestinal lumen and intestinal mucosa of rats treated with \( S. boulardii \) compared with saline-treated controls (Figure 6).

In practice, our data suggest that trehalase deficiency is more common than believed and that in patients with \( \alpha,\alpha \)-trehalase deficiency oral treatment with lyophilized \( S. boulardii \) could increase intestinal trehalase activity and could be beneficial in improving their symptoms related to trehalase intolerance.

A similar situation has been observed and published [21] for patients with sucrase-isomaltase deficiency because the yeast supplies around 800 units sucrase per gram of lyophilized preparation [7]. Although isolated primary or congenital \( \alpha,\alpha \)-trehalase deficiency (0-2 U/g protein) appears to be rare [4], the true incidence of secondary forms of \( \alpha,\alpha \)-trehalase deficiency and partial deficiency remains unknown but could be more frequent than...
expected, especially in patients with dyspepsia caused by smoking [21], food intolerance, coeliac disease [2,3], irritable bowel syndrome or chronic enterocolopathies [2,3].

It is noteworthy that \(\alpha,\alpha\)-trehalase from \(S.\ bouardii\) exhibits sequences highly conserved between species such as the sequence from neutral \(S.\ cerevisiae\) which was used in the present study. Partial purification of the enzyme of \(S.\ bouardii\) exhibited a \(V_{\max}\) and a \(K_m\) close to neutral \(\alpha,\alpha\)-trehalase of \(S.\ cerevisiae\). The same similarity was noticed for the two subunits evidenced on SDS gels of ~82 kDa of mol mass. The mol weight of the unprocessed precursor of neutral \(\alpha,\alpha\)-trehalase of \(S.\ cerevisiae\) is 89,675 kDa [18,19] while the unprocessed precursor of human \(\alpha,\alpha\)-trehalase monomer is 66,595 kDa with a pI of 5.34 [23,24].

Huang et al. [18] have shown that in many organisms trehalase has a critical function in preserving membrane structure and fluidity during dehydration/rehydration. In \(S.\ cerevisiae\), hydrolysis of trehalose depends on both neutral trehalase and acid trehalase (Ath1). Ath1 resides and functions in the vacuole and appears to catalyse the hydrolysis of extracellular trehalose [18,19].

In a recent study of 64 subjects that were submitted to an oral load test of 25 g trehalose, Arola et al. [2] found that 19 of them experienced clear symptoms of dyspepsia and were considered as trehalase-intolerant subjects. Subjects with poor tolerance were best differentiated from tolerant subjects by changes in breath gases (hydrogen and methane) and by the duodenal ratio of trehalase to sucrase activity. Our data are in agreement with this and show that the ratio of trehalase/sucrase is a much better indicator of trehalase deficiency because sucrase activity tends to be stable while lactase activity is variable. Thus, the lower the ratio of trehalase/sucrase, the greater the likelihood that \(\alpha,\alpha\)-trehalase is severely deficient. The correlation between serum and duodenal trehalase activities was in the order of 0.6. Two subjects out of 64 were found to present total selective trehalase deficiency (3%). Arola et al. [2] concluded that it is obvious that trehalose malabsorption can cause digestive symptoms similar to those of lactase deficiency and intolerance. We also found a low incidence of total selective trehalase deficiency (4 cases, 2%) but 57 patients in 200 presented total or partial trehalase deficiency. Three factors control the genesis of symptoms: 1) the low activity of small-bowel trehalase; 2) the maldigested trehalose that causes osmotic flow into the colon; 3) the ability of the colonic microflora to produce gases that result in abdominal distension, flatus and eructation.

Further studies are warranted in the Caucasian population to record the incidence of subjects with trehalase deficiency, their related symptoms of trehalase intolerance and to determine whether in these patients oral treatment with \(S.\ bouardii\) could be beneficial for the relief of symptoms caused by trehalase intolerance.

Acknowledgements

We thank Bernard Hublot for helpful comments on the manuscript and the Laboratories Biocodex for financial support.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References