JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Degradation of Phytate by High-Phytase *Saccharomyces cerevisiae* Strains during Simulated Gastrointestinal Digestion

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Hydrolysis of extracellular phytate ($InsP_6$) by high-phytase yeast strains and survival of yeast cells were studied at simulated digestive conditions using yeast peptone dextrose growth medium and wheat gruel as model meals. An in vitro digestion method was modified to better correlate with the gastric pH gradient following food intake in vivo. High-phytase yeast gave a strong reduction of $InsP_6$ (up to 60%) in the early gastric phase, as compared to no degradation by wild-type strains. The degree of $InsP_6$ degradation during digestion was influenced by the type of yeast strain, cell density, and $InsP_6$ concentration. Despite high $InsP_6$ solubility, high resistance against proteolysis by pepsin, and high cell survival, degradation in the late gastric and early intestinal phases was insignificant. Dependency on pH for phytase expression and/or activity seemed thus to be an important limiting factor. Although further studies are needed, our results show the potential of using yeast as a phytase carrier in the gastrointestinal tract.

KEYWORDS: ***Saccharomyces cerevisiae*; yeast; phytase; phytate; $InsP_6$; solubility; in vitro digestion**

INTRODUCTION

Phytate (*myo*-inositol hexakisphosphate, $InsP_6$) is a naturally occurring compound in plants, where it represents the major storage form of phosphorus. Since phytate is an important constituent in seeds, it is abundant in common plant-based foods such as cereals and legumes (*1*). $InsP_6$ has a strong chelating capacity and forms insoluble complexes with divalent minerals such as iron, zinc, and calcium (*2, 3*). Humans, as well as monogastric animals, lack enzymes in the gastrointestinal tract to degrade phytate, and a diet rich in phytate leads to a considerably impaired absorption of dietary minerals (*4*−*6*), which can be of nutritional importance in vulnerable populations. Phytate is also of major concern in animal feeding because of the low availability of its phosphorus, which, together with the subsequent need for phosphate supplement, contributes to a large phosphate excretion to the environment (*7, 8*).

The dephosphorylation of $InsP_6$ is catalyzed by phytases, forming free inorganic phosphate and inositol phosphate esters with a lower number of phosphate groups, with less ability to influence mineral solubility and intestinal uptake (*6, 9*−*11*). Accordingly, improved iron and zinc absorption can be obtained by degradation of $InsP_6$ during food processes such as bread leavening, sourdough fermentation, hydrothermal treatment, and enzyme treatment (*12*−*15*). In addition, foods with high phytase activity, naturally or induced by processing, may result in degradation of $InsP_6$ in the intestine (*16, 17*) and an improved absorption of zinc, magnesium, and phosphorus (*18*). Furthermore, Sandberg et al. (1996) demonstrated that the inhibitive effect of phytate on iron uptake from single meals in humans was abolished when microbial phytase from *Aspergillus niger* was added to the meal, presumably as a result of intestinal phytate degradation (*19*). Since iron and zinc are absorbed mainly in the upper small intestine, the site and the degree of phytate degradation in the gastrointestinal tract by dietary or exogenous phytase activity are important factors that influence the utilization of these minerals.

Phytase produced by Baker's yeast (*Saccharomyces cerevisiae*) is described in the literature (*20*−*23*). The phytase activity of *S. cerevisiae* has been shown to be at least partly due to the action of the well-studied repressible acid phosphatases (rAPs), which are secreted by the yeast cells and thus enable hydrolysis of $InsP_6$ in growth media (*24*). However, $InsP_6$ hydrolysis by yeast during, for example, bread leavening is relatively low (*21*). This is could be an effect of the expression of the rAPs being repressed by inorganic phosphate (P_i). By deletion of any of the two genes encoding the negative regulators Pho80p and Pho85p in yeast, the P_i repression could be circumvented and the $InsP_6$-degrading ability of *S. cerevisiae* was shown to increase severalfold in both repressing (with P_i) and derepressing (without P_i) media (*25*).

When the goal is expression of phytase activity in vivo by probiotic yeast to gain a trophic effect, i.e., improved mineral absorption, the strain used must survive and be active at conditions in the gastrointestinal tract. It is also important that the organism is safe for human consumption. *S. cerevisiae* has

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10.1021/jf0478399 CCC: $30.25 © 2005 American Chemical Society
Published on Web 05/28/2005

Table 1. Yeast Strains Used in the Experiments

strain	genotype	reference
BY4741	MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*	Euroscarf Acc.No. Y00000
BY80	MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *pho80::kanMX4*	Euroscarf Acc.No. Y01692
BY85	MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *pho85::kanMX4*	EUROSCARF Acc.No. Y02797
YS18	MATα; *his3-11*, *3-15*; *leu2-3*, *2-112*; *ura3Δ5*; *canR*	Sengstag and Hinnen, 1987
YD80	MATα; *his3-11*, *3-15*; *leu2-3*, *2-112*; *ura3Δ5*; *canR*, *pho80Δ::hph*	Veide and Andlid (manuscript)

GRAS status ("generally recognized as safe", as defined by the U.S. Food and Drug Administration) and is thus a promising candidate. Furthermore, *S. cerevisiae* has been shown to be remarkably acid-tolerant, withstanding pH values as low as 1.0 for up to 4 h (*26*). It was also shown to survive the gastric enzymes, bile salts, and pH changes during human digestion. In addition, there were indications that *S. cerevisiae* can out-compete pathogenic yeasts in the intestine (*26*). When assayed at simulated human digestive conditions, a recombinant *S. cerevisiae* strain was shown not only to survive but also to efficiently produce and secrete a heterologous protein and a peptide (*27*).

Here, the potential of high-phytase yeast strains to survive and hydrolyze extracellular $InsP_6$ in complex yeast growth medium and wheat gruel at simulated human digestive conditions was evaluated. An in vitro model was adopted and modified to study the effect of digestive pH conditions, cell density, and substrate concentration on $InsP_6$ degradation. The solubility of $InsP_6$ and the degree of hydrolysis during the different steps of the digestion were also studied.

MATERIAL AND METHODS

Chemicals. Pepsin (P6887), pancreatin from porcine pancreas (P1750), and porcine bile extract (B8631) were purchased from Sigma, and inositol hexaphosphate (sodium phytate, product no. 38045) was purchased from BDH Chemicals Ltd. Chloramphenicol was obtained from Fluka, and Bacto yeast extract and Bacto peptone were obtained from DIFCO. All other chemicals were obtained from Sharlau and Merck. During digestion experiments, freshly prepared pepsin solution (0.3% in 0.1 M HCl with 49 mM NaCl, 12 mM KCl, 10 mM $CaCl_2 \cdot 2H_2O$, 3.5 mM KH_2PO_4, and 2.4 mM $MgCl_2 \cdot 6H_2O$) and a solution with pancreatin (4 g/L) and bile salts (25 g/L) dissolved in $NaHCO_3$ (0.1 M) were used. All glassware and salt solutions were autoclaved at 121 °C before preparation of enzyme solutions.

Yeast Strains and Culturing. Table 1 gives descriptions of the genotypes of the *S. cerevisiae* strains studied and their respective references. The strains used in this study were YS18 and YD80 and the EUROSCARF strains with accession numbers Y00000, Y01692, and Y02797 (hereafter referred to as BY4741, BY80, and BY85, respectively). The EUROSCARF strains were available to us via the Göteborg Yeast Centre (www.gmm.gu.se/gyc). YS18 was constructed by Sengstag and Hinnen (1987) (*28*) and kindly provided by Lennart Adler (Göteborg University, Sweden), and YD80 was constructed by Veide and Andlid (*25*).

Prior to the experiments, all strains were grown overnight in YPD culturing media (1% yeast extract, 2% peptone, 2% dextrose, pH 5.8) at 30 °C in a rotary shaker (200 rpm). To achieve a high ratio of exponentially growing cells, the inocula for the overnight cultures were taken from YPD precultures grown for approximately 6 h at 30 °C in a rotary shaker. Cells were harvested by centrifugation at 4500 rpm (3849*g*) (Multifuge 1 S-R, Heraeus) for 5 min and resuspended in 0.9% NaCl, whereafter the optical density at 610 nm (OD_{610}) was determined. YPD agar plates were used for short-term storage of the strains at 8 °C, while long-term storage was brought out in 20% glycerol at −80 °C.

In Vitro Digestion Procedure. The in vitro digestion was done as described previously (*9, 29*). All solutions were prepared as described by Matuschek et al. (2001) (*30*). Further modifications were here made by the inclusion of a pH gradient in the gastric phase. The gradient was adopted from a program suitable for semisolid meals, used in a computerized in vitro gastrointestinal model described by Minekus et al. (1995) (*31*). The procedure is summarized in **Table 2**.

Table 2. Experimental in Vitro Procedure To Simulate the pH Profile and Enzyme Secretion during Gastrointestinal (GI) Digestion

			addition of enzyme solutions (mL)	
simulated GI part	pH	duration (min)	pepsin[a]	pancreatin/bile[a]
stomach	5.5	10	7.5	
	4.6	10	7.5	
	3.8	10	7.5	
	2.8	20	7.5	
	2.3	20	7.5	
	2.0	20	7.5	
duodenum	5.0	30		13
ileum	6.5	60		

[a] The volume was based on an initial meal volume of 45 mL.

Model meals were either YPD or commercial ready-made wheat gruel (Semper Mild, for children from 6 months old) to which phytic acid solution was added in known concentrations (0−1.25 mM). Chloramphenicol (250 μg/mL) was used in all experiments to diminish bacterial contamination during the digestion procedure. Before initiation of the digestion, yeast cells in 0.9% NaCl suspension were added in an amount corresponding to the desired OD_{610} in the meals; the volume was adjusted to 50 mL with YPD or gruel, and samples were taken to determine $InsP_6$ content (5 mL), $InsP_6$ solubility (1 mL), and colony forming units (cfu) (0.1 mL). The meal was transferred to an Erlenmeyer flask, the pH was manually adjusted, and enzyme solutions were added according to **Table 2**, if nothing else is stated. The pepsin solution was added in equal-sized aliquots at each pH step during the whole gastric phase (a total of 45 mL of pepsin solution). After 90 min, 13 mL of pancreatin and bile solution was added, the pH was adjusted to 5.0, and the digestion mixture was incubated for 30 min. This was followed by adjustment of the pH to 6.5 and an additional incubation for 60 min. During the testing of static gastric pH values, the total portion of pepsin solution (45 mL) was added to the meal and the pH was adjusted to 2 or 3. The sample was incubated for 60 min, followed by the intestinal incubation as described above.

All incubations were done in a rotary incubator (MaxQ 4000, Barnstead Lab-Line) at 37 °C and 220 rpm. The digestions were done in duplicate and, when degradation of $InsP_6$ was to be analyzed in several steps of digestion, separate duplicate flasks were used in which the digestion was terminated at every step in order to avoid extensive reduction of the volumes of the meals.

Determination of Content and Solubility of $InsP_6$. For quantification of $InsP_6$, samples (5 mL) were taken before and after terminated digestion and were acidified with concentrated HCl (220 μL) and extracted during stirring for 3 h. $InsP_6$ was quantified with high-performance ion chromatography (HPIC) as described by Carlsson et al. (*32*). For determination of soluble $InsP_6$, samples (1 mL) were taken during digestion and centrifuged at 13000 rpm (15800*g*) (Eppendorf, 5415 R). The supernatant was decanted and frozen. The supernatant was thawed and centrifuged through Microcon filter devices (Microcon YM-30 catalog no. 42410, Millipore, Bedford, MA) prior to analysis, and the $InsP_6$ content in the filtrate was quantified with HPIC and expressed as soluble $InsP_6$.

Statistical Evaluation. If nothing else is stated, values are presented as the mean ± standard error (SE) of two replicates. Differences in

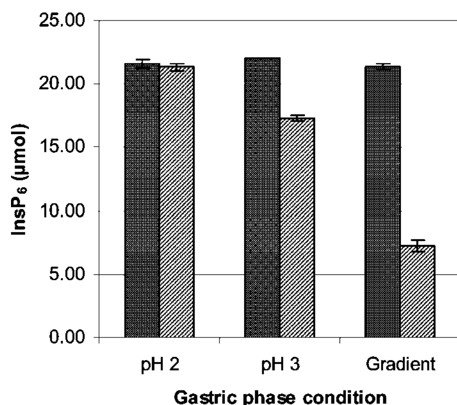

Figure 1. Phytate content in YPD given as μmol in a 45 mL meal volume before (gray bars) and after (striped bars) digestion, showing the effect of different gastric conditions on the level of phytate degradation after completed digestion with static gastric pH set to pH 2 or pH 3 or gastric pH gradient according to **Table 2**. The intestinal phase conditions were the same for all experiments. Values are given as the mean ± SE of two replicates.

mean values were tested by ANOVA using Tukey's HSD multiple-rank test for significant differences between groups (SYSTAT for Windows, SPSS Inc.), and a p value of <0.05 was considered significant.

RESULTS

Development of the in Vitro Method. Several reported in vitro digestion methods are static models that mimic gastric digestion at a certain pH over a defined time period, e.g., pH 2 for 90 min (*9, 29*). In vivo, the gastric pH sharply increases following food ingestion and decreases thereafter to return to the original pH after 3−4 h (*33, 34*). This may be of great importance for the degradation of $InsP_6$ during the gastrointestinal passage, and to acheive a more authentic model, a pH gradient was included in the simulated gastric phase. The different steps of the procedure used in the present study are given in **Table 2**.

A strong degradation of $InsP_6$ was observed (**Figure 1**) when the pH in the gastric phase was adjusted according to the gradient (**Table 2**). In comparison, a static pH of 2 during the gastric phase did not allow any yeast-mediated $InsP_6$ degradation in YPD, and only a minor degradation was observed when the gastric pH was set to 3 (**Figure 1**). In the case of gruel, no degradation was observed in any of the static pH values tested (data not shown).

Capability of Different Strains To Degrade Phytate. To select the most suitable strain for further experiments, three strains modified for improved phytase activity, YD80, BY80, and BY85, were assayed for $InsP_6$ degradation during in vitro digestion of both YPD and gruel. The meals were inoculated with the different strains to a cell density corresponding to an OD_{610} of 5, and $InsP_6$ was added to a concentration of 0.5 mM in the meals. All three modified strains showed a significantly higher capability to degrade $InsP_6$ as compared to their respective wild-types (YS18 or BY4741), which only marginally degraded $InsP_6$ at the present conditions (**Table 3**). Compared to all the other strains, the *pho85*Δ strain, BY85, yielded the highest degradation of $InsP_6$ during in vitro digestion in both YPD and gruel, in which 59% and 40% of the initial $InsP_6$ content were degraded, respectively. *S. cerevisiae* BY85 was therefore selected for all subsequent experiments.

Degradation and Solubility of $InsP_6$ at Specific Points during in Vitro Digestion. The degradation of $InsP_6$ in meals was studied at specific time points during simulated digestion, with 0.5 mM of exogenous $InsP_6$ and an OD_{610} of 5 for BY85 corresponding to 1 mg of dry weight or 5×10^7 cells/mL as the initial levels (**Table 4**). As shown in **Figure 2**, a pronounced degradation of $InsP_6$ occurred in both YPD and gruel during the early gastric phase. After adjustment to pH 2.3 in the gastric phase, no significant change in $InsP_6$ content was observed during the remaining part of the digestion. The relative solubility of $InsP_6$ at each measured point is shown in **Figure 3**. The solubility was between 90% and 100% in YPD at each sampling point except after incubation at pH 6.5, where no soluble $InsP_6$ was detectable. In gruel, the solubility was between 51% and 71% in the late gastric phase (pH 3.8−2.2) and the early intestinal phase (pH 5.0). At pH 2.8 in the gastric phase and again after the early part of the intestinal phase, dips in solubility were observed in the case of gruel. Low-solubility $InsP_6$ was found in the initial step of the digestion (pH 5.5), and no soluble $InsP_6$ was detected in the untreated gruel or in the final step of pH 6.5. Note that these experiments were repeated on two different occasions and that the error bars are given between experiments ($n = 2$) and not between replicates within one experiment.

Table 3. Phytate Degradation (μmol of Degraded $InsP_6$) by Different Yeast Strains ($OD_{610} = 5$) in YPD and Wheat Gruel (0.5 mM Exogenous Phytate[a]) after Completed in Vitro Digestion

	degraded phytate (μmol of $InsP_6$)		degraded phytate (% of initial)	
strain	YPD[b]	gruel[b]	YPD	gruel
YS18	2.1 ± 0.11	1.1 ± 0.05	9	4
YD80	10.9 ± 0.10	5.8 ± 0.0	44	19
BY4741	1.5 ± 0.00	2.7 ± 0.50	6	9
BY80	4.0 ± 0.25	5.6 ± 0.05	18	17
BY85	14.3 ± 0.16	12.6 ± 0.54	59	40

[a] Corresponding to 23 μmol in a 45 mL meal volume. [b] Values are given as the mean ± SE of two replicates.

Degradation in Relation to Initial $InsP_6$ Concentration. The concentration-dependent capacity of BY85 to degrade $InsP_6$ during the simulated digestion was studied using a range of substrate concentrations from 0 to 1.25 mM. As shown in **Table 5**, the absolute amount of degraded $InsP_6$ tended to increase with increasing substrate concentrations. The relative amount of degraded $InsP_6$ was consistently lower in gruel as compared to YPD. The presence of endogenous $InsP_6$ in the gruel resulted, however, in a higher initial total content, of which the endogenous $InsP_6$ constitutes a fraction that is likely to be less available for degradation as compared to the added $InsP_6$. The endogenous phytate was reduced by 18% during digestion when no extracellular phytate was added, however (**Table 5**). As the concentration of added $InsP_6$ increased, the differences between the degradation in gruel and YPD in relation to the initial content declined; i.e., when phytate was added to a concentration of 0.25 mM, the degradation in YPD was as high as 75%, compared to 25% in gruel, whereas at 1 mM added phytate, the relative phytate degradation after digestion was 31% and 27%, respectively.

Effect of Cell Concentration on $InsP_6$ Degradation. Digestions with different amounts of the strain BY85 were done in YPD and gruel containing 0.5 mM $InsP_6$ to assess the effect of cell concentration on $InsP_6$ degradation. **Table 6** shows that increased initial cell concentrations of BY85 coincided with increased $InsP_6$ degradation during digestion in both YPD and gruel. Cells were added in concentrations corresponding to an OD_{610} of up to 7.5, and at this cell density, only 36% of the

Table 4. Total Content and Soluble Phytate (μmol in 45 mL) at Specific Steps during in Vitro Digestion[a]

	YPD μmol of $InsP_6$			gruel μmol of $InsP_6$		
digestive step	total content	soluble fraction	solubility %	total content	soluble fraction	solubility %
initial	23.2 ± 0.02	21.6 ± 0.71	93	32.6 ± 0.23	0	0
post pH 5.5	19.5 ± 0.20	19.9 ± 0.26	102	30.7 ± 0.82	1.0 ± 0.55	3
post pH 3.8	13.7 ± 0.71	12.9 ± 1.47	93	25.7 ± 0.73	18.2 ± 0.83	71
post pH 2.8	7.7 ± 2.49	7.1 ± 2.60	90	20.5 ± 1.81	11.6 ± 1.32	56
post pH 2.0	8.7 ± 2.10	8.2 ± 2.05	94	20.9 ± 1.42	14.3 ± 0.77	68
post pH 5.0	8.0 ± 2.00	7.5 ± 2.10	93	20.8 ± 0.94	10.7 ± 1.14	51
post pH 6.5	7.3 ± 2.00	0	0	20.6 ± 1.64	0	0

[a] Values are given as the mean ± SE of two experiments ($n = 2$) with two replicates each that deviated from the mean by less than 5% for total content and less than 10% for solubility.

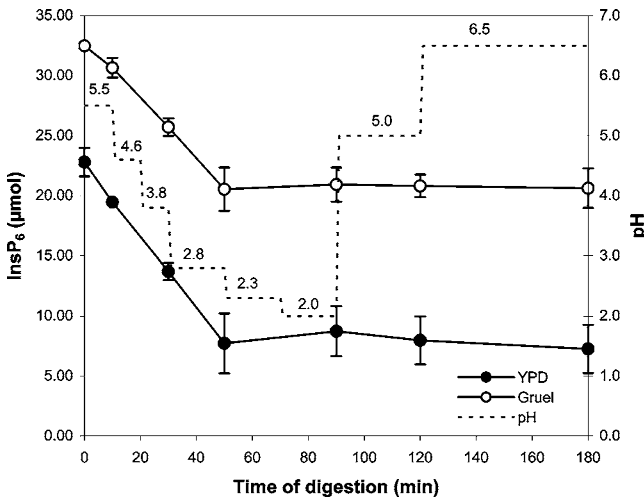

Figure 2. Phytate content in a 45 mL meal volume at specific points during in vitro digestion of YPD and gruel. The pH gradient of time of digestion is shown on the second *Y*-axis. Values are given as the mean ± SE of two separate digestion experiments.

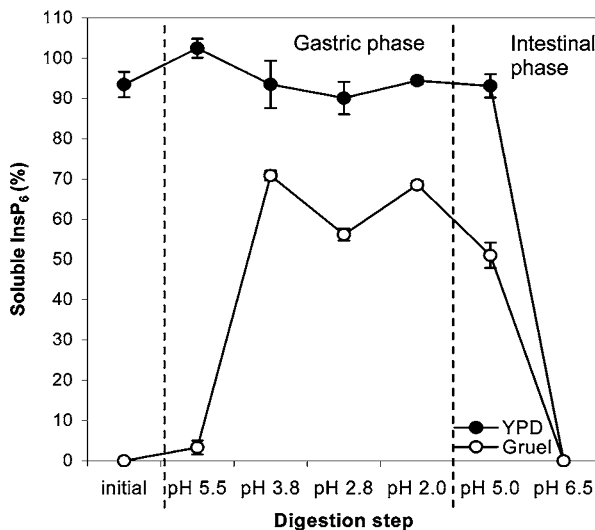

Figure 3. Solubility of phytate in YPD and gruel expressed as percent dissolved $InsP_6$ of total content at specific steps of the in vitro digestion procedure. Initial pH is the pH value of the meals prior to digestion: 5.8 in YPD and 6.5 in gruel. Values are given as the mean ± SE of two separate digestion experiments.

initial $InsP_6$ content in YPD remained after digestion. The same trends were found in gruel (data not shown).

Survival of Yeast Strain BY85 at Digestive Conditions. An important feature of a potential probiotic microorganism is the ability to survive gastrointestinal conditions; hence, the recovery of BY85 in the experiments was monitored. The cell concentrations after digestion, as determined by counting colony forming units (cfu), varied between 106% and 127% of the initial cell concentration in gruel and 70% and 151% in YPD. Altogether, the mean of relative survival of BY85 at an OD_{610} of 5 in YPD and gruel (0.5 mM added $InsP_6$) was 107% ($n = 5$) and 121% ($n = 4$), respectively, which indicates that the yeast cells did survive, and on average they also managed to grow slightly during the digestion. The generation time of BY85 was 2 h and 56 min at optimal conditions (data not shown), however, and since the total time of the simulated digestion procedure was 3 h, a notable growth could not be expected. There was typically a decrease in cell concentration after the gastric phase during the digestions, which was followed by a small increase in cell number after the first part of the intestinal phase (pH 5) in which the conditions of the upper small intestine were simulated.

Table 5. Phytate Degradation as a Function of Different Initial Concentrations of Added Phytate (0–1.25 mM) after Completed in Vitro Digestion in YPD and Wheat Gruel with *S. cerevisiae* BY85 ($OD_{610} = 5$)[a]

	YPD μmol of $InsP_6$				gruel μmol of $InsP_6$			
$InsP_6$ concn,[b] mM	initial[c]	post[c]	degr[d]	degr, %	initial[c]	post[c]	degr[d]	degr, %
0					12.4	10.2	2.2	18
0.25	9.9	2.5	7.4	75	23.5	17.7	5.8	25
0.5	21.8	10.1	11.7	54	37.6	25.5	12.1	32
0.75	32.7	19.9	12.8	39				
1.0	45.5	31.4	14.2	31	60.4	44.3	16.1	27
1.25	58.5	43.0	15.5	26				

[a] Values are given as the mean of two replicates deviating from the mean by less than 5%. [b] Concentration of exogenous $InsP_6$ added to the meals. [c] Analyzed phytate content in a 45 mL meal volume prior to (initial) and after (post) digestion. [d] Degraded phytate.

Table 6. Phytate Degradation as a Function of Different Concentrations of BY85 in YPD (0.5 mM exogenous phytate) after Completed in Vitro Digestion[a]

OD_{610}	initial[b] μmol $InsP_6$	post,[b] μmol $InsP_6$	degr,[c] μmol $InsP_6$	degr, %
1	24.0	19.1	4.9	21
2.5	23.7	16.6	7.07	30
5	24.1	11.1	13.0	54
7.5	24.0	8.6	15.4	64

[a] Values are given as means of two replicates deviating from the mean with less than 5%. [b] Analyzed phytate content in 45-ml meal volume prior to (initial) and after (post) digestion. [c] Degraded phytate.

Reproducibility of Experiments. The degradation level varied to some extent from experiment to experiment because of unavoidable but small variations in the amounts of cells added, the state of the cultures at the start of experiments, and the exact dilution during enzyme addition and pH adjustments. These factors were controlled in order to make them as identical as possible between experiments, and the level of degradation was found to be reproducible. At our "standard condition" (0.5 mM of added $InsP_6$ (23 μmol in 45 mL) and an OD_{610} of 5 for BY85) the remaining $InsP_6$ after complete digestion ranged between 21% and 46% (mean value 39%, $n = 6$) of the initial $InsP_6$ content in YPD and between 59% and 68% (mean value 63%, $n = 5$) in gruel. In total, 14.0 ± 1.05 μmol (11.7−18.7 μmol) of $InsP_6$ of the initial 23 μmol was degraded in 45 mL of YPD during digestion, as compared to 12.6 ± 0.60 μmol (10.6−14.2 μmol) in gruel. The degradation in YPD thus tended to be higher than in gruel, although this was not statistically significant.

DISCUSSION

In the present study, we evaluated the ability of high-phytase strains of Baker's yeast, *Saccharomyces cerevisiae*, to degrade $InsP_6$ during digestion at simulated physiological conditions. *S. cerevisiae* has previously been reported to survive human intestinal passage well (*26*) and to successfully secrete over-expressed proteins during in vitro digestion (*27*), suggesting that yeast can be a suitable carrier of phytase for expression in the intestine. Live yeast cell cultures have previously been found to be ineffective in increasing phosphorus utilization in pigs (*35*). As discussed by Lei et al. (1993) (*36*), this could be a result of insufficient phytase production or poor compatibility with the low pH of the stomach. In the present study, no degradation of $InsP_6$ was found by the wild-type strains, whereas the modified high-phytase strains showed a significant degradation. This indicates the need for high and constitutive phytase production by the yeast strains used.

We assayed three different high-phytase strains, which all resulted in degradation of phytate during digestion and which differed significantly from their respective wild-types. The *pho85*Δ strain, BY85, was the most effective in degrading $InsP_6$ during in vitro digestion and was thus chosen for further study.

A significant degradation of $InsP_6$ occurred during the first part of the gastric phase of the in vitro digestion, whereas there was no further decrease in $InsP_6$ concentration at the pH 2.3 step of the gastric gradient or any of the following steps of the simulated digestion. As will be discussed below, this can be caused by number of factors such as (1) insufficient activity of yeast phytase and/or limited phytase expression at the physicochemical conditions prevailing at the pH 2.3 step and onward, (2) destruction of expressed phytase by proteolytic activity of pepsin, and/or (3) low solubility of $InsP_6$, hindering sufficient enzyme−substrate interactions.

Yeast Phytase Activity. The phytase activity of commercial Baker's yeast has previously been shown to be high at pH 3.5−4.5 (*21*). At pH 5, the relative activity decreased to approximately 50% of the peak activity and decreased sharply to around 25% and lower at pH values below 3.5. In accordance with the pH interval for high yeast phytase activity, the $InsP_6$ degradation in our experiment occurred exclusively in the gastric phase between pH 5.5 and 2.8. The phytase is presumably not active at the low pH conditions in the late gastric phase (pH 2.3 and below), and accordingly, no degradation of $InsP_6$ occurred. However, in the early intestinal phase, conducted at pH 5.0, the $InsP_6$ hydrolysis was not restored, although the phytase should be active at this pH.

Effect of Pepsin Addition on Phytase Activity. Because of the gradual addition, the pepsin concentration increased with time in the gastric phase. This, in combination with the pepsin used being active at pH values below 4.0 (description of product P6887, Sigma), likely resulted in a higher proteolytic activity in the late part of the gastric phase as compared to the early part. Purified yeast phytase produced by *Schwanniomyces occidentalis* has previously been shown to have no activity after an incubation for 30 min with pepsin at pH 2 (*37*). When the incubation was instead done at pH 3 or 4, phytase activity remained to some extent after incubation. Correspondingly, the results of the present study showed that a static gastric pH of 2 did not result in any $InsP_6$ degradation whereas a static gastric pH of 3 resulted in some degradation (**Figure 1**). To control whether this was an effect of the enzymatic degradation of the phytase, the digestive enzymes and bile salts were excluded from the digestive solutions added during digestion in the present study. The resulting $InsP_6$ hydrolysis (data not shown) did not differ from digestion experiments conducted with digestive enzymes; thus, proteolytic degradation of the phytase was not the main cause of the absent $InsP_6$ degradation at the simulated digestive conditions used. Consequently, the results strongly suggest the pH dependency of phytase expression and/or activity as an important limiting factor for the degree of $InsP_6$ hydrolysis during digestion.

Another possible effect of the addition of pepsin could be an increased degradation of $InsP_6$ due to increased $InsP_6$ solubility. This would be a consequence of proteins in some cases strongly interacting with $InsP_6$, decreasing the solubility of $InsP_6$ in, for example, soy protein isolate (*38*). However, we did not see such an effect in our experiments.

Effect of Solubility on $InsP_6$ Degradation. The major part of the phytases (repressible acid phosphatases) produced by yeast is secreted into the periplasmic space but not further through the cell wall to the surroundings (*39*, *40*). Hence, the degradation of $InsP_6$ should mainly occur in the periplasm, to which $InsP_6$ enters through the cell wall by diffusion. The solubility of $InsP_6$ can therefore be an important factor, highly dependent on pH and the presence of minerals and proteins (*3*). Phytate−calcium complexes are, for example, insoluble at pH values above 6.0 (*3*, *38*). Accordingly, no soluble $InsP_6$ was detected in the late gastrointestinal phase (pH 6.5) in any of the meals (**Figure 3**). In YPD, the total fraction of $InsP_6$ was soluble during the whole digestion, with pH 6.5 as the only exception. In gruel, the solubility was found to be relatively high between pH 3.8 and pH 2, as well as in the early intestinal phase (pH 5; **Figure 3**). Nevertheless, there was no degradation in the late gastric phase (**Figure 2**), indicating that the influence of solubility was less important than the effects of low activity of phytase at this low pH. However, the impact of solubility on phytase activity can be discerned in the first step of the gastric phase at pH 5.5. In this phase, there was a small but significant difference in degradation rate between YPD and gruel (**Figure 2**), possibly depending on the significant difference in $InsP_6$ solubility between the two meals at this point (**Figure 3**).

In gruel, despite the relatively small difference in pH, a much higher solubility was observed in the early intestinal phase (pH 5.0) as compared to the early gastric phase (pH 5.5). When the pH dependence of $InsP_6$ solubility in gruel was monitored, by directly adjusting pH with HCl, the solubility was found to be relatively high at pH 5.0, whereas it was zero at pH 6.0 (data not shown). A pH of 5.5 thus ought to be borderline, and it was not surprising to find low solubility at

this step. It is, however, notable that no degradation in the early intestinal phase (pH 5.0) was observed, despite high $InsP_6$ solubility and presumably high phytase activity at pH 5.0. It is possible that the incubation time of this phase was too short to restore the phytase activity after a putative acidic destruction in the previous late gastric phase. The addition of pancreatic enzymes and bile salts at this phase may also impair the phytase expression and/or activity. However, we did not see any differences in the degree of $InsP_6$ degradation when pancreatin and bile salts were excluded (data not shown).

In accordance with previous studies on yeast, a good recovery of the *pho85*Δ strain, BY85, was observed after the in vitro digestion. The strain resisted the acidic conditions during the gastric phase as well as the presence of digestive enzymes. In clinical studies, orally administrated *S. cerevisiae* has also been shown to survive in vivo gastrointestinal passage (*26*, *41*). However, the fecal concentration decreased rapidly after the end of treatment, suggesting that *S. cerevisiae* survives passage through the human gastrointestinal tract but does not permanently colonize the bowel (*41*). This is also consistent with observations made after oral administration of *Saccharomyces boulardii* (*42*), showing that colonization is not required for probiotic effect to take place.

A simple model system was needed to assess the potential of the yeast strains to improve $InsP_6$ degradation in food. Here, YPD and commercial ready-made wheat gruel with a relatively low content of endogenous $InsP_6$ were used, and phytate was added in known concentrations. This enabled us to study the yeast at physiological digestive conditions with minimized diverse counteracting factors such as food matrix, different $InsP_6$ content, and cell structure of the meal, which would need a much more complex in vitro model. If degradation of endogenous $InsP_6$ in, for instance, cereals were to be studied, it would be necessary to include amylase activity and mechanical destruction in the in vitro procedure. To be able to evaluate data, we aimed for an initial concentration of exogenous phytate that was high enough not to be fully degraded during digestion, but still not too high, to risk hiding the degradation or causing substrate inhibition. As seen in **Table 5**, the concentration of exogenous phytate used in this study, i.e., 0.5 mM (23 μmol of $InsP_6$ in 45 mL), yielded a high relative amount of degraded $InsP_6$.

The cell concentration used in this study was in most cases approximately 5×10^7 cells/mL ($OD_{610} = 5$), which corresponds to 1 mg of dry matter/mL or 300 mg of dry yeast in a normal-sized meal of 300 mL. In Sweden, the only yeast used as a probiotic for human use is presently *S. boulardii*, which can be purchased as oral doses of freeze-dried cell culture. For clinical use, the recommended dose for adults is 500 mg twice daily (Precosa, AstraZeneca AB, Sweden). Owing to this, in a future applied situation, a much higher dose of yeast than was used in the present study can be considered, and as shown in **Table 6**, a higher initial cell concentration could result in greater $InsP_6$ degradation.

This report contributes data that can be used for further development of high-phytase strains suitable for $InsP_6$ degradation during the passage through the gastrointestinal tract of humans or animals. It also shows the importance of using a pH gradient instead of static pH for simulating gastric digestion when monitoring the function of microorgansisms. Although further studies are needed, the results show the potential of using yeast as a gastrointestinal phytase carrier.

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Received for review December 22, 2004. Revised manuscript received April 1, 2005. Accepted April 14, 2005. This work was financed by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Project No. 22.2/2002-1458), and the Swedish Nutrition Foundation.

JF0478399