Inositol Hexaphosphate Hydrolysis by Baker's Yeast. Capacity, Kinetics, and Degradation Products

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Phytases hydrolyze *myo*-inositol 1,2,3,4,5,6-hexaphosphate (IP_6), yielding lower inositol phosphates and inorganic orthophosphate. Two commercial strains of baker's yeast (*Saccharomyces cerevisiae*), Y_1 and Y_2, were able to express phytase activity. This was determined by the capacity to grow in a synthetic medium with IP_6 as the sole phosphorus source. IP_6 hydrolysis was rapid for both strains, and after 24 h, all IP_6 was degraded. Control cultures contained inorganic orthophosphate (P_i) and no IP_6. Growth rate in IP_6 medium was for both strains essentially identical to growth in P_i medium, indicating a well-adapted metabolism for utilization of phosphorus from IP_6. There was some difference in growth yield (milligrams of biomass per milligram of glucose) between the two strains: 0.95 (Y_1) and 1.35 (Y_2) in IP_6 medium and 1.03 and 1.35, respectively, in P_i medium. The phytases were of the 3-phytase type, forming mainly DL-Ins(1,2,4,5,6)P_5, DL-Ins(1,2,5,6)P_4, and DL-Ins(1,2,6)P_3.

Keywords: *Phytase; phytate; inositol phosphates; baker's yeast; Saccharomyces cerevisiae*

INTRODUCTION

Phytate (*myo*-inositol 1,2,3,4,5,6-hexaphosphate; IP_6) is ubiquitous in nature and represents the major storage form of phosphorus in plants (Cosgrove, 1980). It has been the object of much research because of its negative effect on mineral bioavailability. In plants, it occurs as a mineral complex, which is insoluble at physiological pH (Erdman, 1979) and is therefore considered to be an antinutrient. In addition, it complexes readily with proteins, thus potentially decreasing the availability of amino acids and the activity of digesting enzymes. Deficiencies of minerals such as iron (Fe) and zinc (Zn), ascribed to high phytate intake, are highly prevalent in vulnerable human populations (Halstedt et al., 1972; Graf, 1986; Ferguson et al., 1989). By reduction of the phytate content in plant foods and feeds the bioavailability of essential dietary minerals can be improved (Sandberg et al., 1989, 1993; Kemme and Jongbloed, 1993).

During food processing, hydrolysis products of phytate, inositol penta- through monophosphates (IP_5−IP_1), can be formed. Thus, foods can contain a mixture of different isomeric forms of inositol phosphates that may interact with each other and other food components. Lower inositol phosphates bind fewer minerals and form weaker mineral complexes (Persson et al., 1998). Concerning the absorption of minerals, previous studies indicate that when added separately to meals, IP_6 and IP_5 but not IP_4 and IP_3 inhibit iron and zinc absorption (Sandberg et al., 1989, 1993; Lönnerdal, 1989; Sandström and Sandberg, 1992). However, when present in a mixture of inositol phosphates, IP_4 and IP_3 may also contribute to the negative effect on iron absorption (Brune et al., 1992) by interactions with the more phosphorylated inositol phosphates through mineral binding (Sandberg et al., 1999).

Phytases are phosphomonoesterases capable of hydrolyzing phytate to free inorganic phosphate (P_i) and lower *myo*-inositol phosphate esters (IP_5−IP_1) and in some cases free *myo*-inositol. Hence, phytases have a potential for improving mineral, phosphorus, and amino acid bioavailability. Phytase activity has been demonstrated to occur in a wide spectrum of organisms, such as plants, microorganisms, and certain animal tissues (Cosgrove, 1980). With respect to position specificity, two kinds of phytases have been recognized. Because they preferentially initiate dephosphorylation of phytate at the 3- and 6-positions of the inositol ring, respectively, they produce different isomers of the lower inositol phosphates. The 3-phytases (EC 3.1.3.8) have been considered to be characteristic of microorganisms and the 6-phytases (EC 3.1.3.26) of the seeds of higher plants (Cosgrove, 1980). However, this is not a general rule, as exemplified by indications of 3-phytase activity in soybean seeds (Phillippy et al., 1988) and 6-phytase activity in *Escherichia coli* and *Paramecium* (Greiner et al., 1993; Kaay and Haasert, 1995).

Microbial phytase activity has been demonstrated in yeast (Nayini and Markakis, 1984; Harland and Frölich, 1989; Türk et al., 1996; Blum et al., 1997). Although phytase of wheat and other plants or fungi has been studied extensively, fairly little is known about the phytase of baker's yeast and the capacity of yeast to hydrolyze phytate during growth.

The aim of this work was to study the phytase activity of baker's yeast (*Saccharomyces cerevisiae*) and to determine whether parameters related to phytate hydrolysis are strain dependent. Two different strains of commercial baker's yeast were investigated for their ability to grow in a minimal medium containing IP_6 as the sole phosphorus source, and the growth was compared with that in an identical medium using inorganic

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phosphate as phosphorus source. Phytase activity was determined by the degradation of IP_6 in the medium. Furthermore, the stereoisomers of the degradation products were studied.

MATERIALS AND METHODS

Yeast Strains. The two strains of *S. cerevisiae* used in this study (Y_1 and Y_2) were obtained from commercial products originating from the Swedish Jästbolaget (Jästbolaget AB, Rotebro, Sweden). One was ordinary fresh yeast (Y_1) and the other was fresh yeast developed especially for sweet dough (Y_2). The strains were maintained on YPD plates (yeast extract, 10 g; peptone, 20 g; glucose, 20 g; and agar, 20 g; in 1 L of water) containing chloramphenicol (250 mg/L) to suppress bacterial contaminants.

Growth Media and Culture Conditions. The growth medium used in all experiments was a modified version of a defined minimal medium developed by CBS (Centralbureau voor Schimmelcultures, Delft, The Netherlands). Either sodium phytate [$C_6H_6(OPO_3Na_2)_6$ (BDH Chemicals, Poole, U.K.; 0.88 mg/mL; experimental cultures] or phosphate [KH_2PO_4 (Merck, Darmstadt, Germany); 3.5 mg/mL; control cultures] was used as phosphorus source, according to the concentrations in the original CBS medium. Phytate-containing cultures also contained KCl (3 mg/mL) to compensate for the potassium present in the inorganic phosphate but not in the phytate. In all experiments 2% (w/v) glucose was used as the carbon and energy source. Experimental cultures (100 mL) were inoculated with primary cultures grown for ~20 h in 20 mL of CBS media of the same compositions as described above. To stabilize the pH in the media, a buffer was used [succinic acid (Merck)/NaOH, pH 5.3]. Media were autoclaved at 120 °C for 20 min with the exception for the heat sensitive phytate and vitamin solutions, which were sterilized by filtration through a 0.2 μm membrane (Microgon, Inc.). The cultures were grown at 30 °C in Erlenmeyer flasks (250 mL) using a rotary shaker at 200 rpm. The growth of the yeast cells was monitored as optical density at 610 nm (OD_{610}) using a spectrophotometer (Hitachi, model U-100).

For each yeast strain phytate-containing cultures and control cultures were grown in duplicates, a total of eight cultures. A sample was removed from each culture at the time intervals indicated in Figure 1 (1−2 h). Means and standard deviations in growth rate (μ) and specific IP_6 hydrolyzing capacity (PHC) for the yeast strains were compared.

Sample Preparation, Inositol Phosphates. Five milliliters of growth medium was withdrawn at intervals during the growth (0, 3, 6, 9, 12, and 24 h) and centrifuged for 5 min at 5000 rpm to remove the cells. To immediately interrupt further phytase activity, the supernatant (4 mL) was acidified by 1.5 mL of 2 M HCl, yielding a 0.5 M solution with respect to HCl and pH ~0.3 and frozen. A reference sample for identification of peaks was prepared by dissolving 1.5 g of sodium phytate in 100 mL of 0.5 M HCl. The solution was reflux boiled for 12 h and evaporated to dryness, and 100 mL of water was added to the hydrolyzed sample.

Inositol Phosphate Analysis. Inositol phosphate content in the culture supernatant was determined by the HPIC method of Skoglund et al. (1997, 1998) with some modifications. The chromatograph consisted of a biocompatible HPLC pump (Waters model 626, Waters Associates, Milford, MA), flow rate = 0.8 mL/min, equipped with a 50 μL injector loop, an HPIC PA-100 analytical column (4 × 250 mm), and a PA-100 (4 × 50 mm) guard column (Dionex Corp., Sunnyvale, CA). Inositol phosphates were detected after postcolumn reaction, at which time the inositol phosphates complex with Fe, by using UV detection (Waters 486, tunable absorbance detector). Absorbance was monitored at 290 nm. IP_3−IP_6 were eluted with a gradient of 5−95% HCl (1.0 M) in conjugation with water. The eluants were combined according to a programmed linear gradient and with the proportions listed in Table 1. The reagents were pumped, at a flow rate of 0.4 mL/min, through a postcolumn reaction pump (Knauer, K-500, Knauer GmbH, Berlin, Germany), and mixed in a postcolumn reactor, with 0.1% $Fe(NO_3)_3 \cdot 9H_2O$ in a 2% solution of $HClO_4$ according to the method of Phillippy and Bland (1988). The combined flow rate was 1.2 mL/min. A mixing tee (Jour Research, Onsala, Sweden) and a reaction coil consisting of a twined Teflon tube (i.d. = 0.2 mm, 4.5 m) optimized with respect to reaction time and avoidance of peak broadening, were applied to obtain sufficient time for a high blending rate.

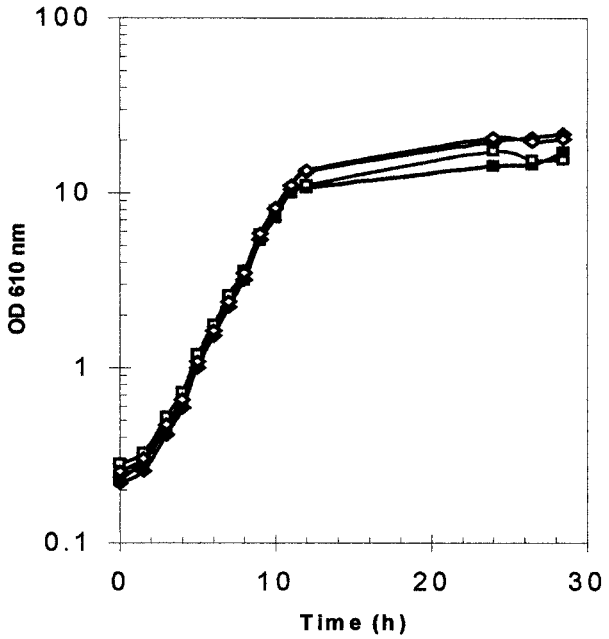

Figure 1. Growth curve of *S. cerevisiae*, baker's strains Y_1 (■) and Y_2 (◆), in synthetic minimal yeast medium containing different phosphorus sources. Growth in IP_6 medium (solid symbols) was compared to growth in P_i medium (open symbols). Data are expressed as means originating from two separate flasks of a representative experiment.

Table 1. Combination of Eluants for the Analysis of Inositol Phosphates

time (min)	HCl (%)	water (%)
0	5	95
15	95	5
18	95	5
19	5	95
25	5	95

RESULTS

In the present study, yeast cells (*S. cerevisiae*, baker's strains) were grown in a CBS medium. Initially, the pH was 5.3 in all cultures. In the phytate-containing cultures phosphorus was present only in the form of IP_6. In the control cultures inorganic orthophosphate was the only source of phosphorus. Both strains of baker's yeast tested were able to develop phytase activity as demonstrated by a rapid growth in IP_6 medium (Figure 1).

Growth Capacity. Both strains showed rapid growth in IP_6 medium (Figure 1). The comparisons of specific growth rates between strains and phosphate sources were performed using data from the respirofermentative phase of growth.

Specifically, the exponential equations used to calculate the growth rates were for all experiments obtained from OD data between times of 1.5 and 10 h (Table 2). This yielded a regression coefficient (R^2) value of 0.99 for all cultures. As an index of the efficiency in utilizing phosphorus from IP_6, the growth rate ratio μ_{pho}/μ_{phy} was

Table 2. Growth Parameters for Yeast Strains (*S. cerevisiae*)[a]

growth parameter	strain Y_1	strain Y_2
μ_{pho} (h^{-1})	0.386 ± 0.001	0.400 ± 0.001
μ_{phy} (h^{-1})	0.389 ± 0.004	0.413 ± 0.001
μ_{pho}/μ_{phy}	0.992[b]	0.968[b]
g_{pho} (h)	1.796 ± 0.003	1.732 ± 0.004
g_{phy} (h)	1.782 ± 0.017	1.678 ± 0.006
g_{pho}/g_{phy}	1.008[b]	1.032[b]
final OD_{pho}	28.1 ± 0.7	36.9 ± 1.6
final OD_{phy}	25.9 ± 0.7	36.8 ± 0.6
final X_{pho} (mg/mL)	20.55[b]	26.98[b]
final X_{phy} (mg/mL)	18.94[b]	26.91[b]
OD_{phy}/OD_{pho}	0.92[b]	1.00[b]
$yield_{pho}$	1.028[b]	1.349[b]
$yield_{phy}$	0.947[b]	1.346[b]

[a] Synthetic medium with phytate (phy) or phosphate (pho) as the source of phosphorus and glucose (2%) as the carbon and energy source. Specific growth rate (μ) and generation time (g), in the respirofermentative phase. Optical density (final OD) and biomass formed (final *X*) at cessation of growth. Parameters are calculated on the values from two separate incubations (mean ± SD). [b] Calculated from the mean values.

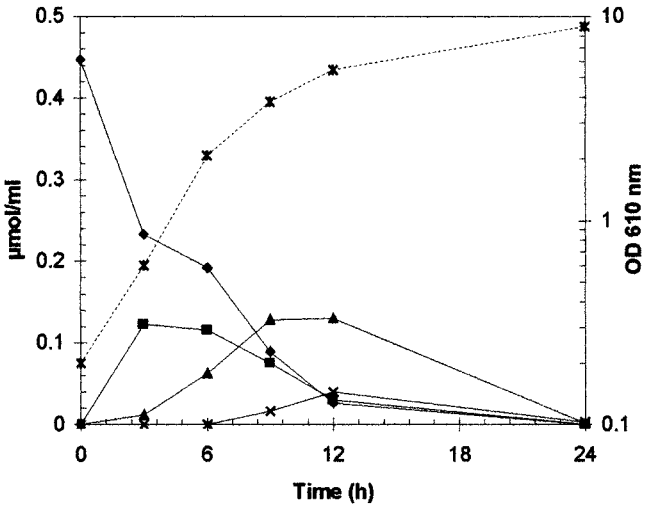

Figure 2. Growth of *S. cerevisiae*, baker's strain Y_1, as compared to the reduction of IP_6 and the formation of lower inositol phosphates in the medium as a function of time. Cell density is presented as OD_{610nm} (dotted line), and the concentration of inositol phosphate (IP_6, ◆; IP_5, ■; IP_4, ▲; IP_3, ×) as μmol/mL. For the study of the breakdown profile, the initial concentration IP_6, 0.45 μmol/mL, was used.

used, where μ_{phy} is the growth rate in phytate medium and μ_{pho} is the growth rate in phosphate medium. Table 2 demonstrates μ_{phy}/μ_{pho} of the yeast strains at pH 5.3 and 30 °C. It is shown that both strains could utilize phosphorus from phytate without detectable loss in growth rate as compared to the growth rate in phosphate medium (rate ratio close to 1). However, there was a small difference in growth rate between the strains on both types of medium. The growth yields as expressed in final optical density per gram of glucose consumed show some interesting differences. Strain Y_2 grew to a denser culture than Y_1 in both phytate and phosphate media (see Table 2). Furthermore, the strains differed in the relative yields between the two phosphorus sources. Y_1 had a final yield in IP_6 medium that was only 92% of the yield in P_i medium, whereas for Y_2, the growth yields were fairly similar in the two media.

Phytate Degradation. IP_6 degradation was complete in 24 h. Figure 2 depicts the degradation of IP_6 and the formation of lower inositol phosphates (IP_5–IP_3) during the first 12 h of cell growth (strain Y_1). The stepwise procedure of dephosphorylation is demonstrated by the subsequent increase in IP_5, IP_4, and IP_3, respectively. However, it is clear from the data that degradation of IP_5, IP_4, and IP_3 starts before IP_6 is depleted, demonstrating a competition for different IP substrates by the yeast enzyme. Hence, the rate of IP_6 degradation will decrease with time as seen by the data in Figure 2.

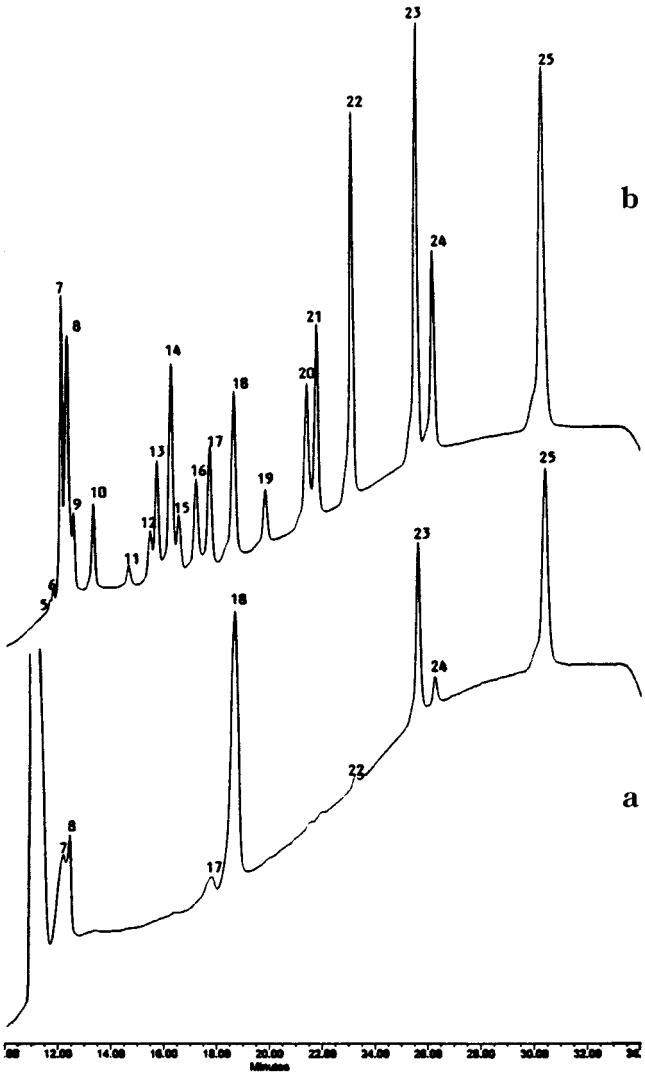

Figure 3. Chromatographic profile of inositol phosphates present in (a) the medium, after 12 h of growth of *S. cerevisiae*, strain Y_1, at 30 °C, pH 5.3, and (b) reference IP_6 hydrolyzed by HCl. Unidentified peaks are assigned an asterisk (*). Peaks: (5) *; (6) *; (7) DL-Ins(1,2,4)P_3, Ins(1,3,4)P_3; (8) DL-Ins(1,2,6)P_3, Ins(1,2,3)P_3; (9) DL-Ins(1,4,5)P_3; (10) DL-Ins(1,5,6)P_3; (11) Ins(4,5,6)P_3; (12) Ins(1,2,3,5)P_4; (13) DL-Ins(1,2,4,6)P_4; (14) DL-Ins(1,2,3,4)P_4; (15) Ins(1,3,4,6)P_4; (16) DL-Ins(1,2,4,5)P_4; (17) DL-Ins(1,3,4,5)P_4; (18) DL-Ins(1,2,5,6)P_4; (19) Ins(2,4,5,6)P_4; (20) DL-Ins(1,4,5,6)P_4; (21) Ins(1,2,3,4,6)P_5; (22) DL-Ins(1,2,3,4,5)P_5; (23) DL-Ins(1,2,4,5,6)P_5; (24) Ins(1,3,4,5,6)P5; (25) IP_6.

Degradation Products. The chromatographic profiles of the reference sample and of the growth medium after 12 h (Y_1) of growth are demonstrated in Figure 3 (the chromatographic profile was similar for Y_2). The phytases of the yeast strains studied proved to be of the 3-phytase type. The main IP_6 degradation products were DL-Ins(1,2,4,5,6)P_5, DL-Ins(1,2,5,6)P_4, and DL-Ins(1,2,6)P_3.

Table 3. PHC for Two Strains of Baker's Yeast[a]

strain-dependent parameter	strain Y_1	strain Y_2
ΔIP_6 (μmol/mL)	0.798 ± 0.001	0.814 ± 0.001
ΔX (mg/mL)	10.5 ± 0.2	13.0 ± 0.4
$\Delta IP_6/\Delta X$ (μmol/mg)	0.076[b]	0.063[b]
PHC [μmol of IP_6/(g of yeast × h)]	8.6 ± 0.2	7.1 ± 0.2

[a] IP_6 degradation (ΔIP_6) and biomass formation (ΔX) per milliliter during the first 12 h of growth (respirofermentative phase). Data are mean ± SD from two separate cultivations. [b] The ratio was calculated from the mean values.

Specific Phytate-Hydrolyzing Capacity (PHC). The degradation of IP_6 by the two strains was studied as a function of time and as a function of biomass formed during the respirofermentative phase of growth. To relate the data to the actual amount of fresh baker's yeast, OD_{610} of a series of yeast solutions with known biomass concentrations (wet weight) was measured. The result was used to create a standard curve, and the concentration of yeast in the experimental cultures could be approximated from OD_{610} using the equation $y = 1.369x - 0.035$ ($y = OD_{610}$, $x =$ mg of yeast/mL; $R^2 = 0.9957$).

The PHC for each strain was calculated as the amount of IP_6 degraded per hour and milligram of yeast biomass. The data are presented in Table 3. Despite the similarity in growth parameters, there was a difference in PHC between the strains. Strain Y_1 had a slightly higher PHC than Y_2 (Table 3).

DISCUSSION

To increase mineral bioavailability, it is desirable to reduce the IP_6 content in bread. IP_6 hydrolysis during dough fermentation can be optimized using yeast strains with high phytase activity. In the present study, yeast strains (*S. cerevisiae*, baker's strain) were cultured on CBS medium in which phosphorus was present only in the form of IP_6 to determine the inherent IP_6-hydrolyzing capacity under optimal conditions. One important objective was to investigate whether different strains of *S. cerevisiae* have different capacities and kinetics in IP_6 breakdown.

For two reasons the specific growth rates between strains and phosphate sources were compared using data from the respirofermentative phase of growth:

(i) The respirofermentative phase with fermentation of glucose to ethanol represents the type of metabolism prevailing during many applied situations such as bread-making.

(ii) In the respirofermentative phase, growth rate is not successively decreasing as a result of oxygen limitation, which often is the case in dense cultures during the late respiratory phase of growth.

Other studies demonstrate phytase activity in baker's yeast (Blum et al., 1997; Türk et al., 1996; Harland and Frölich, 1989; Nayini and Markakis, 1984). In agreement with this both strains of baker's yeast tested in the present study were able to develop phytase activity. Besides, we have also demonstrated strain-dependent differences in PHC. Baker's yeast strains expressed a growth in IP_6 medium essentially identical to their growth in phosphate medium (Figure 1 and Table 2), indicating an efficient and well-adapted metabolism for utilization of phosphorus hydrolyzed from IP_6. The obtained generation time of 1.7 h is normal for unrestricted growth by *S. cerevisiae*. Furthermore, for both strains the overall shapes of the growth curves were similar between the two media (Figure 1).

The transition occurred simultaneously in both media, showing that no large amount of extra energy was required for hydrolyzing IP_6 during the first growth phase.

Both strains demonstrated an efficient IP_6 degradation. However, a small difference indicated that strain Y_1, as calculated per time and biomass, was superior to Y_2 in IP_6 dephosphorylation. This indicates that the expressions of phytase genes are different between the two strains and/or the phytase produced by Y_1 has a higher specific activity, and this phytase may hence be the more interesting enzyme for future studies. The PHC for Y_1 was 8.6 [μmol of IP_6/(g of yeast × h)] during the first 12 h of growth (Table 3). Provided the same rate of hydrolysis, 50 g of yeast would thus degrade $50 \times 8.6 = 430$ μmol in 1 h. A bread containing in total roughly 6 mmol of IP_6 would at that rate decrease to 5.57 mmol (7.2%) in IP_6 content as a result of *yeast* phytase activity solely. Although a significantly larger decrease than previously detected during bread-raising assigned to *yeast* phytase activity [(Türk et al., 1996), the main degradation of IP_6 was demonstrated to be a result of activity by endogenous *plant phytases* present in the flour], this is, however, not enough and two conclusions may be drawn: (1) The yeasts have an inherent higher specific capacity for IP_6 degradation than occurs during bread-raising, showing that conditions for *yeast* phytase expression are not optimal during bread-making. This heightens the need for (2) more efficient strains to be screened for or developed, which makes sense because the yeast component seems more feasible to optimize than the endogenous phytase component.

The phytases of the two yeast strains studied were shown to be of the 3-phytase type, with very isomer-specific chromatographic profiles.

It is of nutritional significance to degrade inositol phosphates in cereal products during food processing, for example, bread-making. Yeast phytase of a high activity, or a yeast strain with a high expression of phytase during fermentation, could be one way of achieving extensive phytate degradation. Previous studies indicate that baker's yeast does contribute to phytate degradation during bread-making. However, this contribution was evidently extremely small (Türk et al., 1996). Therefore, it was logical to ask whether the yeast PHC is poor or if conditions during bread fermentation disfavor phytase expression.

The data presented here show that, provided optimal conditions, *S. cerevisiae* show excellent growth by hydrolyzing IP_6 for its phosphorus requirements. Hence, the enzyme as such is not the problem; it is rather a matter of increasing the expression. We are at present addressing the latter possibility.

ABBREVIATIONS USED

IP_3−IP_6, *myo*-inositol tri- through hexakisphosphate; Ins, the numbering of the *myo*-inositol phosphates may be clockwise (L) or counterclockwise (D) (Ins is an accepted NC−IUB abbreviation for *myo*-inositol with the numbering of the D configuration unless the prefix L is explicitly added); 3-phytase (EC 3.1.3.8), *myo*-inositol hexakisphosphate + H_2O → D-*myo*-inositol 1,2,4,5,6-pentakisphosphate + orthophosphate (starts

the dephosphorylation of IP_6 in the C-3 position, when counted in the D-configuration); 6-phytase (EC 3.1.3.26), *myo*-inositol hexakisphosphate + H_2O → L-*myo*-inositol 1,2,3,4,5-pentakisphosphate + orthophosphate (starts the dephosphorylation of IP_6 in the C-6 position, when counted in the L-configuration (= C-4 position when counted in the D-configuration)); *X*, biomass (mg/mL); Y_1, Y_2, strains of *Saccharomyces cerevisiae* obtained from commercial products [fresh yeast (Y_1) and fresh yeast developed especially for sweet dough (Y_2)]; YPD plates, solid culture medium for yeast cells; CBS medium, liquid culture medium for yeast cells; OD_{610}, optical density measured by spectrophotometry at 610 nm; *g*, generation time (h); μ, specific growth rate (h^{-1}); PHC, specific phytate-hydrolyzing capacity [μmol of IP_6/(g of yeast × h)]; HPIC, high-performance ion chromatography.

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