CHARACTERIZATION OF EXTRA-AND INTRACELLULAR PHYTASES FROM PROLIVERATING YEAST DURING TEMPEH PRODUCTION

Sutardi1

ABSTRACT
Proliferating yeast species such as Endomycespora burtonii, Candida dididiasme and Candida tropicalis, produced both extra- and intracellular phytases during tempeh (soaking of soybeans) production. The enzymes were isolated from growth media and the cultured yeast and partially purified by r-czone fractionation. Extracellular phytase activities were higher than that of the intracellular enzymes. The extra- and intracellular phytases of E. burtonii, C. dididiasme and C. tropicalis acted over a narrow pH ranging at 2.2 - 4.8 and the maximum activity at temperatures between 45° - 70°C. Estimated Kd values for extra- and intracellular phytases from yeast were within the range 0.5 - 3.1 x 10-14 M. The Vmax values were within the range 0.004 - 0.19 mmol P1 liberated/ h/mg enzyme for the extra- and intracellular phytases of E. burtonii, C. dididiasme and C. tropicalis. The activation energy for hydrolysis of phytic acid by extra- and intracellular phytases of E. burtonii, C. dididiasme and C. tropicalis were calculated by the Arrhenius equation being 7,100; 9,100; 13,000, 5,600, 7,300 and 21,700 cal/mole, respectively.

Key words: characterization, Endomycespora sp., Candida sp., phytase, tempeh.

INTRODUCTION
Tempeh is a traditional Indonesian fermented food made from soaked and cooked soybeans inoculated with a mold, usually of the genus Rhizopus. Soaking of dry soybeans usually forms an integral part of processing method for tempeh production. Eventually, micro-organism such as proliferating yeast during soaking of soybeans has been expected to produce either extra- or intra-cellular phytase that are able to reduce phytic acid content of beans as suggested by Jaffe (1991).

Phytase, or myo-inositol hexaphosphatase phosphatydrolase (EC 3.1.11.4 or 3.1.3.26) (UB, 1979), which can hydrolyze phytic wad into myo-inositol and orthophosphate (P1), has been isolated from several different micro-organisms including bacteria (Powar and Jaganathn, 1967), yeast (Nayini and Markkin, 1981; Yannings et al., 1999) and moulds (Saharmadji, 2000; Sutardi and Bockle, 1983), and also some legumes seeds (Sutardi and Bockle, 1986).

The yeast phytase is produced by the most common contaminating microorganisms during tempeh production. The enzyme was isolated, purified and characterized as part of a study to establish whether phytase reduction during tempeh production is the result of physical treatments such as soaking, boiling and steaming or due to hydrolysis by phytases produced by yeast contaminating during soaking of raw soybeans overnight.

MATERIALS AND METHODS
Organisms
Yeast that grew well during the soaking of soybeans were isolated and identified as Endomycespora burtonii, Candida dididiasme and Candida tropicalis. The yeast cultures were transferred to a Malt Extract Agar (MEA) slant, incubated for 48 h at 30°C and maintained for further experiments.

Cultivation of ergasterms
Loop of pure culture yeast was inoculated into 10 ml Malt Extract Broth (MEB) in a test tube and incubated for 16 h at 30°C, and then transferred into 100 ml of MEA in a 300 ml erlenmeyer flask, and incubated in an orbital shaking incubator for 24 h at 30°C and 200 rpm. The number of cells in every step of cultivation was enumerated by a spread plate method on MEA. Cultivations were carried out in triplicate on at least two different preparations of culture.

Harvesting of ergasterms
Pure yeast cultures were harvested by centrifugation for 30 min at 4°C and 15,000 x g. Supernatants were collected and used as the source of extracellular phytase, while precipitates were used as the source of intracellular phytases.

Crude enzyme preparation
Extracellular enzyme supernatant obtained from centrifugation of growth media was concentrated by freeze drying (-35°C, 3.75 kPa), thawed, and dialysed for 48 h at 4°C against 0.01 M tris-maleate buffer pH 6.5, then subjected to acetone fractionation.

Intracellular phytase, prepared from the precipitate, was treated with doubly distilled water and then centrifuged for 15 min at 10,000 x g. Precipitate was suspended with 20 ml 24 CaCl2/10 g cells (wet basis) in a 50 ml glass tube, and 10 g of glass beads (0.45 - 0.50 mm diameter) was added. Yeast cells were disrupted in a vortex mixer at maximum speed (Hazen and Cutler, 1982). The disruption periods lasted 20 s each, and were followed by rechilling in an ice bath for 1 min. This was repeated until a residual disruption time of 2 min was accumulated (Richards and

1 Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta, Indonesia.
Takai, 1979). The suspension was centrifuged for 30 min at 4°C and 15,000 x g. Precipitate was washed with 10–20 ml 2% CaCl₂ and disruption was repeated as above and centrifuged for 30 min at 4°C and 5,000 x g. The supernatants were collected and subjected to acetone fractionation. The proportion of cell breakage was determined using method described by Hana and Cutler (1982).

**Acetone fractionation**

Acetone fractionation was carried out as for mould phytase fractionation (Satardi and Buckle, 1988). The acetone purified enzymes were used for subsequent experiments.

**Enzyme assay**

Extracellular yeast phytases were assayed in stopped test-tubes at 70° ± 1°C and 55° ± 1°C for E. bentonii, C. daldenii and C. tropicalis, respectively. Intracellular yeast phytases were assayed at 65° ± 1°C, 55° ± 1°C and 45° ± 1°C for E. bentonii, C. daldenii and C. tropicalis, respectively, by measuring the rate of increase of Pi using the arsenic acid method (Wasternack and Olsen, 1965). The reaction mixture had a total volume of 1.20 ml and contained 0.20 ml of 0.1 M acetate buffer, pH 4.2 and 4.8 for extra- and intracellular phytases of C. daldenii and C. tropicalis, respectively, except pH 3.9 and 3.3 for the extra- and intracellular phytases of E. bentonii. 0.15 ml 8 mM sodium phytate previously adjusted to pH as mentioned above with 1 N HCl; 0.20 ml enzyme preparation and 0.65 ml double distilled water. The final concentration of phytate was 1.0 mM. The reaction mixture was incubated for 30 min at temperature as mentioned above. After incubation, samples were withdrawn, deproteinized by adding 0.80 ml 10% TCA and total protein was analyzed by Lowry method (Lowry et al., 1951). Further procedure and determinations of phytase activity by following the procedure developed by Satardi and Buckle (1988).

**Effect of pH**

The effect of pH on phytase activity was determined in 0.1 M acetate buffer with final concentration 1.0 mM sodium phytate as substrate, incubated for 30 minutes at temperatures specified above. Mixtures were adjusted to pH 3.2 – 4.3 and 2.4 – 4.1 at 0.1 pH intervals for extra- and intracellular phytases of E. bentonii, and to pH 3.6 – 4.7 and 4.4 – 5.3 at 0.1 pH intervals for extra- and intracellular phytases of C. daldenii and C. tropicalis, respectively.

**Effect of temperature**

Phytase assay mixtures were incubated for 30 minutes over the temperature range 35° – 70°C at 5 degree C intervals and 70° – 90°C at 10 degree C intervals.

**Effect of substrate concentration**

Optimum substrate concentration for phytase activity was determined by following the procedure as described by Satardi and Buckle (1988) except that sodium phytate concentration were from 0 – 0.25 mM at 0.05 mM intervals and from 0.25 – 2.00 mM at 0.25 mM intervals. K₉₀ and Vₜ₉₀ values were estimated by the method of Lineweaver and Burk (1934).

**Rate of denaturation**

Partially purified enzyme was heated at 70° and 80°C, 60° and 75°C, and 55° and 65°C for extracellular phytases of E. bentonii, C. daldenii and C. tropicalis, respectively, and at 65° and 75°C, 55° and 65°C, and 45° and 55°C for intracellular phytases of E. bentonii, C. daldenii and C. tropicalis, respectively, for 0 – 60 min at intervals of 10 min, cooled, sodium phytate added to a final concentration of 1.0 mM and phytases assayed by using the procedure modified by Satardi and Buckle (1988). The energy of inactivation and temperature coefficients were estimated by the Arrhenius equation.

**Thermal inactivation for phytase**

Enzymes in buffer solution were heated in a waterbath for 10 min over the temperature range 40° – 100°C, cooled, sodium phytate added to a final concentration of 1.0 mM and phytases were assayed by following the procedure developed by Satardi and Buckle (1988).

**Effect of incubation time and temperature**

Phytase activity was assayed during incubation of reaction mixtures at 40°, 50°, 60°, 70°, and 80°C for extracellular phytases of E. bentonii and C. daldenii, at 30°, 40°, 45°, 50°, and 60°C for extracellular phytase of C. tropicalis, at 40°, 50°, 55°, 60°, 65°, and 70°C for intracellular phytases of E. bentonii and C. daldenii, and at 30°, 40°, 45°, 50°, and 55°C for intracellular phytase of C. tropicalis for times from 30 to 180 min at 30 min intervals. The energy of activation for phytase was estimated from the Arrhenius equation.

**RESULTS AND DISCUSSION**

Production of phytase

E. bentonii, C. daldenii and C. tropicalis grown in Malt Extract Broth (MEB) medium produced both extracellular and intracellular phytases. Relatively little is known about the production of yeast phytases; in one survey of a range of microorganisms for production of extracellular phytase, none of the yeasts examined was found to produce phytase (Shieh and Waal, 1968). Nagy and Markakis (1984) reported that baker's yeast produced an intracellular phytase, but extracellular phytase was not described.

In the present study the partially purified yeast extracellular phytases showed higher total activity than did the intracellular phytases (Table 1). E. bentonii produced significantly higher levels of extracellular phytases, while C. daldenii produced the lowest levels of extra- and intracellular phytases. Wang et al. (1980) reported that some strain of A. oryzae grown in synthetic medium produced higher extracellular phytase activity than intracellular phytase. Some of them produced the same level of enzymes when they were grown in rice medium.

Agritech vol.23 No. 2 yearman 57 - 66
### Table 1. Yield and specific activities during purification of extra- and intracellular yeast phytoases of E. bourbonii, C. didymus and C. tropicus.

<table>
<thead>
<tr>
<th>Purification Stage</th>
<th>Yeast</th>
<th>Intra-</th>
<th>Intercellular</th>
<th>Intercellular Phytoase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total yield (g)</td>
<td>Total Activity (U)</td>
<td>Specific Activity (U/mg)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>Cellulose phospho</td>
<td>E. bourbonii</td>
<td>1729.5</td>
<td>270.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>C. didymus</td>
<td>1729.5</td>
<td>270.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>C. tropicus</td>
<td>1729.5</td>
<td>270.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Phosphate</td>
<td>E. bourbonii</td>
<td>1729.5</td>
<td>270.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>C. didymus</td>
<td>1729.5</td>
<td>270.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>C. tropicus</td>
<td>1729.5</td>
<td>270.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Freeze-drying</td>
<td>E. bourbonii</td>
<td>185</td>
<td>491</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>C. didymus</td>
<td>185</td>
<td>491</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>C. tropicus</td>
<td>185</td>
<td>491</td>
<td>2.6</td>
</tr>
<tr>
<td>Alcohol by-products</td>
<td>E. bourbonii</td>
<td>176</td>
<td>491</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>C. didymus</td>
<td>176</td>
<td>491</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>C. tropicus</td>
<td>176</td>
<td>491</td>
<td>2.6</td>
</tr>
<tr>
<td>Aeration</td>
<td>E. bourbonii</td>
<td>34</td>
<td>114</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>C. didymus</td>
<td>34</td>
<td>114</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>C. tropicus</td>
<td>34</td>
<td>114</td>
<td>3.3</td>
</tr>
<tr>
<td>Dialysis and</td>
<td>E. bourbonii</td>
<td>28</td>
<td>22.7</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>C. didymus</td>
<td>28</td>
<td>22.7</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>C. tropicus</td>
<td>28</td>
<td>22.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

One unit of enzyme is that amount of protein that can liberate 1 mole P1 per min from the respective substrate under experimental conditions. Phosphate was assayed by using sodium phosphate (1 mM) as substrate.

### Isolation and purification of phytoases

Both extra- and intracellular phytoases were isolated from growth media and yeast cells, respectively. Yield and specific activities of the phytoases at various stages of purification are presented in Table 1. At each stage of purification, specific activities of the intracellular phytoases were higher than those of the extracellular enzyme except in the culture filtrates (before purification), and after freeze-drying and dialysis of phytoases from C. tropicus.

The extracellular phytoases of E. bourbonii, C. didymus and C. tropicus were purified 72, 46 and 17 fold, respectively, with recoveries 72.6, 26.3 and 21.9%, respectively. The intracellular phytoases were purified by 71, 33 and 59 fold, with recoveries of 40.9, 56.7 and 25.3% for E. bourbonii, C. didymus and C. tropicus, respectively. The purification achieved by enzyme fractionation was substantially higher than that obtained by freeze-drying and dialysis.

### Effect of pH

The effect of pH on both extra- and intracellular yeast phytoases is shown in Figure 1. The pH optima of extra- and intracellular phytoases from C. didymus and C. tropicus were 4.2 and 4.8, respectively, while pH optima of extra- and intracellular phytoases from E. bourbonii were 3.9 and 3.5, respectively. The pH optima for all species of yeast examined characterize the enzymes as acid phosphohydrolases as reported by Wang et al. (1989) for phytoase from A. oryzae. Various pH optima have been reported by a number of investigators for the cellular phytoase from microorganisms (Rayadi, 1988). In the present study, extracellular phytoase activities increased moderately before and after the optimum pH. The extracellular phytoase activity of C. tropicus was destroyed above pH 5.3 with about 75% loss of activity.

Intracellular phytoase activity of E. bourbonii increased slightly before the optimum pH but was diminished moderately at pH above the optimum pH, while intracellular phytoase activity of C. didymus increased sharply before and after the optimum pH. More than 70% and 90% loss of activity was recorded when the pH decreased to below 3.7 and rose to above 4.4, respectively.

Intracellular phytoase activity of C. tropicus increased moderately up to pH 4.7 and sharply up to the pH optimum; above pH 4.9 the enzyme activity decreased moderately. Above pH 5.3 more than 80% loss of activity occurred. The pH optima of the three yeast phytoases were within the range (pH 2.2 - 3.6) reported for microbial phytoases (Graf, 1986).

![Figure 1. The pH activity profile of extra- (A) and intracellular (B) phytoases from E. bourbonii (E1), C. didymus (C1, C3) and C. tropicus (C2). Vertical bars indicate range of duplicate determination on each of 2 enzyme preparations.](image-url)
Effect of temperature

The temperature-activity profile of the extracellular phytases from *E. coli*, *C. dactyloides* and *C. tropicalis* show maximum activity at 70°, 55°, and 55°C, respectively, while intracellular phytases had maximum activity at 65°, 55°, and 45°C, respectively (Figure 2). Inactivation of the extracellular phytases commenced above 80°C for *E. coli* and *C. dactyloides*, and above 70°C for *C. tropicalis*. Incubation above these temperatures caused loss of activity of over 90%, and at 100°C the enzymes were completely destroyed.

As the temperature increased from 37°C to the optimum temperature, extracellular phytase activities increased about 5 folds, while intracellular phytase activities increased about 5, 10, and 25 folds for *E. coli*, *C. dactyloides* and *C. tropicalis*, respectively. Inactivation of the intracellular phytase commenced at 70°C for *E. coli* and *C. dactyloides*, at 80°C the activities were completely destroyed. The intracellular phytase of *C. tropicalis* was inactivated at about 60°C, and at 100°C caused 90% loss of activity. The temperature optima found for the yeast phytases were within the range (40° - 70°C) reported by previous investigators (Sutaré, 1988).

![Figure 2](image)

**Figure 2.** The temperature-activity profile of extracellular (A) and intracellular (B) phytases from *E. coli* ( ), *C. dactyloides* ( ), and *C. tropicalis* ( ). Vertical bars indicate range of duplicate determinations of each of 2 enzyme preparations.

Effect of substrate concentration

The effect of substrate concentration on phytase activity is illustrated in Figure 3. Similar to phytase from various sources, the activities of extra- and intracellular yeast phytases were inhibited by high concentrations of phytic acid. Extracellular phytase from *E. coli* and *C. dactyloides* were inhibited by concentrations of phytic acid greater than 1.0 mM, while the extracellular phytase from *C. tropicalis* was inhibited by a phytic acid concentration greater than 1.75 mM. Like the extracellular phytases, the intracellular phytases were inhibited also at phytic acid concentrations greater than 1.0 mM.

Both extra- and intracellular phytases of *C. dactyloides* had maximum activity at 1.0 mM substrate concentration under optimum assay conditions, above which the activity marginally decreased.

The initial reaction velocity was proportional to substrate concentration up to 1.0 mM. Above 1.0 mM, activity increased less rapidly and the rate of increase in activity was no longer proportional to the substrate concentration. Except for the phytases from *C. dactyloides*, typical rectangular hyperbolic curves were obtained, following the Michaelis-Menten's equation.

Nair and Markakis (1984) reported that baker's yeast was inhibited at a phytic acid concentration of 1.0 mM and at 10 mM the activity was completely terminated. Significant inhibition at 0.40 - 0.75 mM phytic acid concentration was observed for mould phytase of *Rhizopus oligosporus* (Sutaré and Buckle, 1988).

Agron. J. 23 No. 2 halma 37 - 66
Based on the results shown in Figure 3, a double reciprocal plot of substrate concentration against reaction rate produced $K_m$ values of $1.4 \times 10^{-6}$, $3.1 \times 10^{-6}$ and $1.0 \times 10^{-5}$ M for extracellular phytases from E. buttontii, C. diddensiiae and C. tropicalis, respectively. For the intracellular phytases from E. buttontii, C. diddensiiae and C. tropicalis, $K_m$ values were $0.5 \times 10^{-5}$, $6.7 \times 10^{-5}$ and $0.6 \times 10^{-5}$ M, respectively (Figure 4). The $K_m$ values for extracellular and intracellular phytases were within the range ($10^{-5} - 10^{-7}$ M) reported by Palmer (1981). The $K_m$ values of extracellular phytases were relatively higher compared with the $K_m$ values for the intracellular phytases, indicating weak affinity between the former enzymes and the substrate.

$V_{max}$ values, determined by the same manner as for the $K_m$ values, were 0.19, 0.65 and 0.02 mole P, liberated/min/ml enzyme for extracellular phytases from E. buttontii, C. diddensiiae and C. tropicalis, respectively, and were 0.09, 0.004 and 0.004 mole P, liberated/min/ml enzyme, respectively for intracellular phytases of E. buttontii, C. diddensiiae and C. tropicalis, respectively. Extracellular yeast phytases had higher $V_{max}$ values than did intracellular yeast phytases.
Figure 4. Lineweaver-Burk plot for activity of extra- (A) and intracellular (B) phytoases from *E. hartrofti* (*n,t*), *C. diddensiae* (*Δ*,*v*) and *C. tropicallis* (*∪*,*v*). Data obtained from. Figure 3 are plotted as reciprocal of the initial rate (1/V, minμmole⁻¹) versus reciprocal of the substrate concentration (1/S, mM⁻¹).

$V_{max}$ of extra- and intracellular phytoases from *E. hartrofti* were higher compared to those $V_{max}$ of extra- and intracellular phytoases from *C. diddensiae* and *C. tropicallis*. Comparison with the $V_{max}$ of other microbials phytoases cannot be made because of the differences in the units used (Sutardi, 1988). In the present study $V_{max}$ of yeast phytoase is lower than the $V_{max}$ of mould phytoase (0.076 - 0.34 μmol P per min per ml enzyme) (Sutardi and Boccié, 1988).

Rate of denaturation

Extra- and intracellular phytoase activities of *E. hartrofti*, *C. diddensiae* and *C. tropicallis* remaining after heating at temperatures as specified are shown in Figure 5. The extracellular phytoase of *E. hartrofti* shows a large difference in rate of denaturation at 70°C and 80°C. Heating this phytoase at 80°C for 10 min reduced phytoase activity to very low levels compared to the another two extracellular phytoases. Incubation at temperature above the optimum temperature increased the rate of denaturation of the enzymes, as did prolonged heating.

The inactivation-time relationship deviated significantly from linearity, and two inactivation rate constants (k) can be estimated by the procedure described above. Extracellular phytoases from *E. hartrofti*, *C. diddensiae* and *C. tropicallis* had inactivation energy of 78,800, 70,500 and 18,600 cal/mole, respectively. The temperature coefficient of inactivation ($Q_{10}$) over the intervals of 70° - 80°C, 60° - 70°C, and 55° - 65°C for extracellular phytoases of *E. hartrofti*, *C. diddensiae* and *C. tropicallis*, respectively, were 25, 25 and 2. Inactivation energies and $Q_{10}$ of the extracellular phytoases were relatively high compared with those for various extracellular phytoases reported by previous investigators (Sutardi, 1988).
Intracellular phytases from E. hurstonii, C. diddensiae and C. tropicais had activation energies of 6,300, 12,600 and 14,900 cal/mole, respectively. The temperature coefficients of inactivation were 15, 2 and 2, respectively. The relatively high values in activation energies and $Q_10$ of the extracellular phytases from E. hurstonii and C. diddensiae cannot be explained.

Thermal inactivation

Figure 5 shows the effect of heating for 10 min on thermal inactivation of yeast phytases. Incubation of the heated enzyme preparation for 10 min at up to 60$^\circ$C marginally depressed extracellular phytase activity of E. hurstonii, but increased significantly the activity of extracellular phytase from C. diddensiae; the extracellular phytase of C. tropicais increased in activity at 50$^\circ$C, but decreased at temperatures of 65$^\circ$C and above.

All extracellular phytase activities decreased sharply at temperatures above 60$^\circ$C, while at temperature above 80$^\circ$C all enzyme activities were practically absent. Heating of the extracellular phytase from C. diddensiae at temperature below 60$^\circ$C or below its optimum temperature (55$^\circ$C) appears to activate rather than inactivate the phytase. A similar activation effect occurred when the extracellular phytase of C. tropicais was heated for 10 min at 50$^\circ$C (below its optimum temperature, 55$^\circ$C), but not for extracellular phytase of E. hurstonii which has a higher optimum temperature (i.e. 70$^\circ$C). The intracellular phytase of E. hurstonii was highly activated on heating for 10 min at temperature up to 70$^\circ$C indicating the stability of the enzyme. In contrast, activities of intracellular phytases from C. diddensiae and C. tropicais were significantly decreased at temperature as low as 40$^\circ$C. At temperature at or above 80$^\circ$C intracellular phytase activities were almost completely destroyed, and no activity remained for the intracellular phytase of E. hurstonii. Thus heating at 80$^\circ$C or above completely destroyed both extracellular and intracellular yeast phytases, in agreement with the observation by Yamada et al. (1968) that the phytase from Aspergillus terreus was effectively destroyed at 80$^\circ$C.

Figure 5. Rate of denaturation of extracellular (A) and intracellular (B) phytases from E. hurstonii (●), C. diddensiae (△), and C. tropicais (□). Vertical bars indicate range of duplicate determination on each of 2 enzyme preparations.

Agricull. Vol. 25 No. 3. 1966: 57-66
Figure 6. Thermal inactivation of extra- (A) and intracellular (B) phytases from *E. burtonti* (a, i), *C. diidens* (B, c) and *C. trichodes* (C, m). Vertical bars indicate range of duplicate determination on each of 2 enzyme preparations.

Table 2. Effects of incubation time and temperature on the liberation of P, from phytate by extra- and intracellular phytases from *E. burtonti*. 

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Extraocular phytase activity at incubation time (min)</th>
<th>Intracellular phytase activity at incubation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>40</td>
<td>0.03 (0.0)</td>
<td>0.04 (0.0)</td>
</tr>
<tr>
<td>50</td>
<td>0.06 (0.0)</td>
<td>0.04 (0.0)</td>
</tr>
<tr>
<td>55</td>
<td>0.09 (0.0)</td>
<td>0.09 (0.0)</td>
</tr>
<tr>
<td>60</td>
<td>0.14 (0.0)</td>
<td>0.14 (0.0)</td>
</tr>
<tr>
<td>65</td>
<td>0.19 (0.0)</td>
<td>0.19 (0.0)</td>
</tr>
<tr>
<td>70</td>
<td>0.24 (0.0)</td>
<td>0.24 (0.0)</td>
</tr>
</tbody>
</table>

* Activity defined as μmole P liberated per ml acetone purified enzyme.
* Mean ± s.d. of duplicate determination on each of two enzyme preparations.

Agric Sci Fud 23 No. 2 halatus 57 - 66
Table 3. Effects of incubation time and temperature on the liberation of Pi from phosphate by extra- and intracellular phytases from C. difficile.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Extracellular phytase activity at incubation times (min)</th>
<th>Intracellular phytase activity at incubation times (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.059648</td>
<td>0.061230</td>
</tr>
<tr>
<td>30</td>
<td>0.086960</td>
<td>0.089000</td>
</tr>
<tr>
<td>40</td>
<td>0.115294</td>
<td>0.117594</td>
</tr>
<tr>
<td>50</td>
<td>0.143628</td>
<td>0.145928</td>
</tr>
<tr>
<td>60</td>
<td>0.171962</td>
<td>0.174262</td>
</tr>
<tr>
<td>70</td>
<td>0.199300</td>
<td>0.201600</td>
</tr>
</tbody>
</table>

Activity defined as µmol P liberated per ml sections purified enzyme.

Mean ± s.d. of duplicate determinations on each of two enzyme preparations.

Table 4. Effects of incubation time and temperature on the liberation of Pi from phosphate by extra- and intracellular phytases from C. tropicus.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Extracellular phytase activity at incubation times (min)</th>
<th>Intracellular phytase activity at incubation times (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.059648</td>
<td>0.061230</td>
</tr>
<tr>
<td>30</td>
<td>0.086960</td>
<td>0.089000</td>
</tr>
<tr>
<td>40</td>
<td>0.115294</td>
<td>0.117594</td>
</tr>
<tr>
<td>50</td>
<td>0.143628</td>
<td>0.145928</td>
</tr>
<tr>
<td>60</td>
<td>0.171962</td>
<td>0.174262</td>
</tr>
<tr>
<td>70</td>
<td>0.199300</td>
<td>0.201600</td>
</tr>
</tbody>
</table>

Activity defined as µmol P liberated per ml sections purified enzyme.

Mean ± s.d. of duplicate determinations on each of two enzyme preparations.

The highest activities were observed at 40°, 60° and 55°C for extracellular phytases of E. coli, C. difficile and C. tropicus, respectively, and at 65°, 55° and 45°C for the intracellular phytases from E. coli, C. difficile and C. tropicus, respectively, which are consistent with previous observation in the present study (Figure 2).

From the data in Table 2, 3 and 4, the activation energies for the hydrolysis of phytic acid by extra- and intracellular phytases were calculated by the Arrhenius equation and the results are presented in Figure 7. Extracellular phytases of E. coli, C. difficile and C. tropicus had activation energies of 7,100, 9,100 and 13,000 cal/mole, respectively, while the intracellular phytases of these organisms had activation energies of 5,600, 7,100 and 21,700 cal/mole, respectively.

Figure 1. Arrhenius plots for estimation of activation energy from the hydrolysis of phytic acid by extra- (A) and intracellular (B) phytases from E. coli, C. difficile and C. tropicus (°C). Initial rate (v) estimated from data of Table 2, 3 and 4; T = absolute temperature.
CONCLUSION

E. turtoni, C. diddensiae and C. tropicales grown in MEB medium produced both extra- and intracellular phytases. The extracellular phytases of E. turtoni, C. diddensiae and C. tropicales were purified 72, 46 and 17 fold, respectively with recoveries 72.6, 26.3 and 21.9%, respectively. The intracellular phytases were purified by 71, 33 and 59 fold, with recoveries of 40.9, 56.7 and 25.5% for E. turtoni, C. diddensiae and C. tropicales, respectively.

The optimum pH of extra- and intracellular phytases from C. diddensiae and C. tropicales were 4.2 and 4.8, respectively, while pH optimum of extra- and intracellular phytases from E. turtoni were 3.9 and 3.2, respectively. The temperature-activity profile of the extra- and intracellular phytases were at 45° - 70°C for E. turtoni, C. diddensiae and C. tropicales.

Estimated Kₐ values for extra- and intracellular phytases from yeast were within the range 0.5 – 3.1 x 10⁸ M. The Vₕ values were within the range 0.004 – 0.19 μmole P liberated/min/mg enzyme for the extra- and intracellular phytases of E. turtoni, C. diddensiae and C. tropicales.

Extracellular phytases of E. turtoni, C. diddensiae and C. tropicales had activation energies of 7,100, 9,100 and 13,000 cal/mole, respectively; while the intracellular phytases of these organisms had activation energies of 5,000, 7,100, and 21,700 cal/mole, respectively.

ACKNOWLEDGEMENT

The author is grateful to Prof. K.A. Buckle for his supervision and guidance in the research and preparing of this manuscript for publication, and to R.B.K. Vidyowidjono who has prepared and donated yeast pure culture of E. turtoni, C. diddensiae and C. tropicales.

REFERENCES


