Characterization of glycosidases produced by
Pseudomonas fluorescens 26

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ABSTRACT

Six synthetic glycosides were used to characterize glycosidic activities of a cell-free filtrate of Pseudomonas fluorescens 26. The filtrate was prepared after growing the bacterium in glucose-enriched minimal growth broth. Temperature and pH optima for the filtrate were as follows, respectively, β-D-fucosidase: 15°C and 6.0, β-D-mannosidase: 25°C and 6.0, β-D-glucosidase: 25°C and 5.5, β-D-galactosidase: 30°C and 6.5, N-acetyl-β-D-glucosaminidase: 45°C and 7.0, and N-acetyl-β-D-galactosaminidase: 45°C and 6.0. Activation energies of β-D-galactosidase, β-D-mannosidase, β-D-glucosidase and N-acetyl-β-D-glucosaminidase were 25.4, 12.3, 9.8 and 6.0 Kcal/mol, respectively. β-D-fucosidase and N-acetyl-β-D-galactosaminidase appeared to have non-Arrhenius behavior, so activation energies were not calculated for them. All six glycosidases were heat-sensitive to conditions of pasteurization of milk.

Glycosidases enzymes release, by hydrolysis, sugars complexed with proteins, lipids and other substances. Microorganisms producing these enzymes may use them to gain a source of energy. Removal of a carbohydrate moiety may also, by eliminating steric hindrance, provide access by other hydrolases to the remainder of the molecule. Thus production of glycosidases by microorganisms in foods is potentially significant.

The production of glycosidases by several psychrotrophic bacteria, including Pseudomonas fluorescens 26, was reported previously (16). Although several researchers (1,5,8,13,20) studied glycosidases from non-psychrotrophic bacteria, there are few references on characterization of glycosidases produced by psychrophiles. Among the few hydrolases previously studied, β-glucosidase of P. fluorescens W was purified partially and characterized by Guffanti and Corpe (11).

The purpose of the present research was to characterize the glycosidase activities of P. fluorescens 26 grown in minimal growth broth. Parameters studied were optimal temperature and pH for glycosidic activity, activation energy (Ea), activity at 20°C and stability of glycosidases to pasteurization temperatures.

MATERIALS AND METHODS

Preparation of the crude enzyme of Pseudomonas fluorescens 26

Minimal growth broth (MGB) was prepared from 7% (v/v) each of dehydrated sodium citrate (2 mM), ammonium sulfate (9.2 mM), glucose (2 mM), and a solution of salts which contained heptahydrated magnesium sulfate (2.1 mM), dihydrated calcium chloride (2.3 mM) and sodium chloride (0.086 M); 4% (v/v) citric acid (0.1 M); dibasic sodium phosphate buffer at pH 6.0; and 68% (v/v) distilled water. The broth was inoculated with P. fluorescens 26 (originally isolated from milk) from a freshly prepared trypticase soy agar (TSA) slant. The transmittance at 520 nm of the uninoculated MGB was adjusted to 100%, and the broth was inoculated until its transmittance had decreased to 90%. After 2 d of incubation at 20°C, cells were removed by centrifugation at 39,000 × g for 15 min. The supernatant fluid (crude enzyme) was protected from further bacterial growth with 100 International Units of Combicin (Sigma Chemical Co.) per millilitre. A fresh supply of crude enzyme was prepared for each experiment.

Optimal temperature for glycosidase activity

Six different synthetic substrates, p-nitrophenyl-glycosides (PNP-glycosides), were separately dissolved in MGB buffered at pH 6.0. A concentration of 2 mM of PNP-β-D-fucoside, PNP-β-mannoside, PNP-β-D-glucoside, PNP-β-D-galactoside, PNP-N-acetyl-β-D-glucosaminidase and PNP-N-acetyl-β-D-galactosaminidase was used. Test tubes containing 1 ml of MGB with synthetic substrate and 0.1 ml of crude enzyme were separately incubated at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55°C for 48 h. After incubation, 1 ml of glycine (pH 10.5) was added to stop the reaction, and absorbance of the free p-nitrophenol (PNP) was determined at 400 nm in a dual-beam Beckman spectrophotometer, model 25. Controls without crude enzyme were concurrently tested for each temperature and synthetic substrate. The experiment was twice replicated with duplicates within each replicate.

Optimal pH for glycosidase activity

The same six synthetic substrates and experimental procedure used in the test for optimal temperature were used in this experiment. The MGB, containing 2 mM p-nitrophenyl-glycoside substrates and 0.1 ml of crude enzyme, was buffered at pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5 with citric acid (0.1 M) and dibasic sodium phosphate (0.2 M). There were two replicates with duplicate samples in each.

Activation energy of glycosidases

Absorbance data from the tests for optimal temperature were used to
determine the activation energy \((E_a)\) of glycosidases of \(P. fluorescens\) 7. Reaction rates \((k)\), mean absorbancies divided by the incubation time \((48\, h)\), were calculated at different temperatures for each glycosidase, as follows: for the \(\beta\)-D-fucosidase at 10 and 15°C; for the \(\beta\)-D-mannosidase and B-D-galactosidase at 10, 15 and 20°C; for \(\beta\)-D-galactosidase at 10, 15, 20 and 25°C; and for both \(\beta\)-D-hexosaminidases at 10, 15, 20, 25, 30, 35 and 40°C.

Mean reaction rates were used to obtain the regression equation for the theoretical straight line resulting from the plot of the decimal logarithm of \(k\) versus the inverse of the absolute temperature, \((K^{-1})\). The slope of this line was equal to \(-E_a/2.303R\). Since \(R\), the Gas Constant, was equal to 1.987 cal/mole°K, \(E_a\) was readily calculated for each glycosidase.

### Activity of glycosidases at 20°C

One unit of glycosidic activity was defined as the amount of glycosidase of \(P. fluorescens\) 7 which released 1 pico-mole of PNP per hour of incubation at 20°C. Absorbance values from the test for optimal temperature obtained at 20°C were converted to units of activity using a standard curve of PNP concentration, the sample volume (2.1 ml) and the incubation time (48 h). The average of four determinations was reported as units of activity of each glycosidase at 20°C.

A standard curve for absorbance vs. concentration of PNP was prepared in MGB using concentrations of PNP ranging from \(10^{-9}\) M to \(10^{-7}\) M. Absorbance was determined with a Bausch and Lomb Spectronic-20 at 400 nm. The regression equation was calculated using the mean of absorbance readings from duplicate samples.

### Stability of glycosidases to pasteurization temperatures

Two portions (1.5 ml each) of the crude enzyme of \(P. fluorescens\) 26 were separately heated in 1.5 x 16 cm test tubes in a water bath to 63°C for 30 min (low-temperature long-time or LTLT pasteurization) and 72°C for 15 s (simulating high-temperature short time or HTST pasteurization). The surface level of crude enzyme in the test tubes was immersed approximately 2 cm below the water level of the bath. The crude enzyme was immediately cooled in an ice bath after the heat treatment. The "come-up-time" to 55°C for each procedure was 1 min. This "come-up-time" is probably longer than that of HTST heating; therefore, the treatment may have been more severe than normal pasteurization. Heated and unheated crude enzyme (10%) were added to each of the six p-nitrophenyl synthetic substrates (2 mM) in glucose-enriched MGB. Samples were incubated for 2 d at the optimal temperature and pH of each glycosidase. These conditions of incubation of MGB, containing the respective p-nitrophenyl-glycosides, were the following: PNP-\(\beta\)-D-fucoside at 15°C and pH 6.0; PNP-\(\beta\)-D-mannoside at 25°C and pH 6.0; PNP-\(\beta\)-glucoside at 25°C and pH 5.5; PNP-\(\beta\)-D-galactoside at 30°C and pH 6.5; PNP-N-acetyl-\(\beta\)-D-glucosaminide at 45°C and pH 7.0 and PNP-N-acetyl-\(\beta\)-D-galactosaminide at 45°C and pH 6.0. Tests were made in duplicate. Absorbance values were determined at 400 nm.

### Statistical analyses

Statistical analyses (19) were done after modifying the data by the square root transformation, \((X + 1)^{1/2}\). This transformation was used to stabilize the variance which was statistically proportional to the mean.

Experimental designs of the tests of optimal temperature and pH for glycosidic activity were two-way replicated 6 x 11 and 6 x 7 factorials, respectively, while the tests of activation energy and activity at 20°C had a simple two-way classification design.

Analysis of variance was used to study the effects of independent variables (replicates, treatments and glycosidases) and to test for their possible interaction with the dependent variable (absorbance of free PNP, activation energy or activity at 20°C of glycosidases). Duncan's Multiple Range and Least Significant Difference (LSD) tests were used to determine differences among means when \(F\) values were significant \(\left(P<0.05\right)\).

### RESULTS AND DISCUSSION

#### Crude enzyme of \(P. fluorescens\) 26

Crude enzyme of \(P. fluorescens\) 26 was prepared by inoculating the MGB to the extent that the growth medium before incubation had a transmittance value of about 90%. Thus the population of \(5 \pm 1 \times 10^9\) cells per ml of broth after incubation was sufficient to produce amounts of enzyme typical of the late log or early stationary phase of growth. These amounts were adequate to release significant p-nitrophenol on incubation for 48 h when 10% of the crude enzyme was added to the solution containing the substrate.

#### Optimal temperature for glycosidase activity

Temperature had its greatest effect on the activity of \(\beta\)-D-galactosidase (Fig. 1), which was highly active at 30°C. In contrast, curves depicting activities against the five other substrates were relatively flat. Individual temperature optima ranged from 15 to 45°C.

\(\beta\)-D-fucosidase had no activity above 25°C. The activity of this glycosidase at 15°C was significantly higher than that at 5, 20 and 25°C, but it was not different from that at 10°C.

Based only on activity at the various temperatures, \(\beta\)-D-mannoside and \(\beta\)-D-glucoside appeared to be hydrolyzed by a single enzyme. There were no significant differences in activity of \(\beta\)-D-mannosidase from 10 to 30°C or of \(\beta\)-D-glucosidase from 20 to 30°C.

Since the activity of crude enzyme in the presence of PNP-\(\beta\)-D-mannoside and PNP-\(\beta\)-D-glucoside differed sig-
nificantly at 25°C from the activity against PNP-β-D-fucoside, temperature optima with these substrates indicated the data represented activities of at least two different glycosidases.

A third distinctive glycosidase of *P. fluorescens* 26 appeared to be β-D-galactosidase (lactase), which had significantly higher activities from 25 to 35°C compared with activities of the other glycosidases. Bouvy (2) found that *Saccharomyces lactis* lactase was also most effective at 35°C with 0-nitrophenyl-β-galactoside as a substrate. Morisi et al. (17) reported that at pH 7.2 yeast β-galactosidase had an optimal temperature of 37°C, while that of *Escherichia coli* showed the highest glycosidic activity at 55°C. In a study of whole cells (13), the highest activity of β-galactosidase from *Propionibacterium shermanii* was found at 52°C. Finocchiaro et al. (6) obtained slightly different results with lactase from *Kluyveromyces lactis*. Investigating the immobilization of this glycosidase on alumina, they found that the optimal temperature of free β-galactosidase was 45°C.

Comparing activities in the presence of the two PNP-N-acetyl-β-D-hexosaminides, we found no significant differences between 35 and 55°C. N-acetyl-β-D-glucosaminidase had no differences in activity from 40 to 50°C, while activity of N-acetyl-β-D-galactosaminidase did not differ significantly from 20 to 45°C. There was no conclusive evidence from optimal temperatures that these were two different glycosaminidases.

Therefore, *P. fluorescens* 26 appeared to produce at least four different glycosidases based on optimal temperature for glycosidase activity.

The optimal pH for β-D-glucosidase activity was 5.5, slightly lower than that reported by Garibaldi and Gibbins (8) for β-D-glucosidase from *Erwinia herbicola* Y46.

The highest β-D-galactosidase activity was obtained at pH 6.5. This coincides with several reports (6,10,14,17) in which lactase from different sources was studied biochemically. Hartley et al. (13) found a slightly higher optimal pH (7.5) for β-galactosidase from *P. shermanii* in a whole-cell assay.

The activity of N-acetyl-β-D-glucoaminidase from *P. fluorescens* 26 was not statistically different from pH 6.0 to 7.0. Yem and Wu (20) stated that *E. coli* K-12 N-acetyl-β-D-glucoaminidase, after being purified to near homogeneity, showed a pH optimum of 7.7.

On the other hand, the N-acetyl-β-D-galactosaminidase activity presented a lower optimal pH range (5.5-6.5) than N-acetyl-β-D-glucosaminidase. Activities of both N-acetyl-β-D-hexosaminidases were significantly different at all pH levels, as were the activities of β-D-mannosidase and β-D-glucosidase. Therefore, based on optimal pH for glycosidase activity, *P. fluorescens* 26 appeared to produce at least five different glycosidases.

Activation energy of glycosidases

Activation energy (Ea) is the amount of energy required to bring all the molecules in one mole of a substance at a given temperature to the activated state (15). The Ea can be calculated from the slope of the line given by the Arrhenius equation. If the pre-exponential parameter A of that expression is temperature-dependent, the line is curved and the Ea value is not constant. This was possibly true for N-acetyl-β-D-galactosaminidase (Fig. 3) and β-D-fucosidase (not shown).

![Figure 2. Mean absorbancies of p-nitrophenol liberated by glycosidases of Pseudomonas fluorescens 26 between pH 4.5 and pH 7.5.](image)

**Optimal pH for glycosidase activity**

Maximal activities of glycosidases of *P. fluorescens* 26 were observed from pH 5.5 to pH 7.0 (Fig. 2). The activity of β-D-fucosidase did not vary significantly in the 4.5-7.5 pH range. Moreover, this activity was not statistically different from that of β-D-mannosidase in the same pH range. However, the activity of β-D-mannosidase was about twice higher than that of β-D-fucosidase at pH values of 6.0 and 6.5.
TABLE 1. Activation energies (Kcal/mole) of glycosidases of Pseudomonas fluorescens 26.

<table>
<thead>
<tr>
<th>Glycosidase</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Mean</th>
<th>Transformed mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-mannosidase</td>
<td>12.45</td>
<td>12.20</td>
<td>12.33</td>
<td>3.64</td>
</tr>
<tr>
<td>β-D-glucosidase</td>
<td>9.11</td>
<td>10.55</td>
<td>9.83</td>
<td>3.22</td>
</tr>
<tr>
<td>β-D-galactosidase</td>
<td>25.38</td>
<td>25.31</td>
<td>25.35</td>
<td>5.13</td>
</tr>
<tr>
<td>N-acetyl-β-D-glucosaminidase</td>
<td>6.29</td>
<td>5.70</td>
<td>6.00</td>
<td>2.64</td>
</tr>
</tbody>
</table>

*Means of duplicate determinations.

\[ \text{Transformed mean} = \left\{ \sum_{i=1}^{4} (A_i + 1)^{1/2} \right\} + 4. \]

1-4Means with different superscripts are significantly different (P<0.05).

TABLE 2. Units of activity* at 20°C of glycosidases of Pseudomonas fluorescens 26.

<table>
<thead>
<tr>
<th>Glycosidase</th>
<th>Replicate 1</th>
<th>Replicate 2</th>
<th>Mean</th>
<th>Transformed mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-D-fucosidase</td>
<td>3.7</td>
<td>2.3</td>
<td>3.0</td>
<td>2.01</td>
</tr>
<tr>
<td>β-D-mannosidase</td>
<td>4.7</td>
<td>4.0</td>
<td>4.4</td>
<td>2.31</td>
</tr>
<tr>
<td>β-D-glucosidase</td>
<td>5.7</td>
<td>5.6</td>
<td>5.7</td>
<td>2.61</td>
</tr>
<tr>
<td>β-D-galactosidase</td>
<td>22.1</td>
<td>36.0</td>
<td>29.1</td>
<td>5.53</td>
</tr>
<tr>
<td>N-acetyl-β-D-glucosaminidase</td>
<td>16.2</td>
<td>18.0</td>
<td>17.7</td>
<td>4.32</td>
</tr>
<tr>
<td>N-acetyl-β-D-galactosaminidase</td>
<td>29.6</td>
<td>29.7</td>
<td>29.7</td>
<td>5.53</td>
</tr>
</tbody>
</table>

*One unit of activity was the amount of glycosidase that released 1 pmole of PNP per hour of incubation at 20°C.

\[ \text{Transformed mean} = \left\{ \sum_{i=1}^{4} (A_i + 1)^{1/2} \right\} + 4. \]

1-3Means with different superscripts are significantly different (P<0.05).

Generally, the parameter A is independent of the temperature, thus the Arrhenius plot is a straight line as shown for N-acetyl-β-D-glucosaminidase in Fig. 3. Table 1 gives the two replicated values, the overall means and the statistically transformed means of Ea obtained for each of four glycosidases that responded linearly.

The Ea of P. fluorescens 26 β-D-galactosidase (25.35 Kcal/mole) was similar to that (26 ± 3 Kcal/mole) reported by Fink and Angelides (5) in their study of β-galactosidase from E. coli K-12 at low temperatures with o-nitrophenyl-β-D-galactoside as a substrate. On the other hand, Hustad et al. (14), using the same bacterial β-galactosidase, found that the Ea was 15.2 Kcal/mole. In a similar study, Sharp et al. (18) reported the value of 17.5 Kcal/mole for the β-galactosidase from E. coli ML-308.

The Ea of N-acetyl-β-D-glucosaminidase (6.0 Kcal/mole) of P. fluorescens 26 was lower than that (10.7 ± 0.8 Kcal/mole) determined by Donovan and Hansen (3) in egg white, using p-nitrophenyl-β-D-glucosaminide as a substrate.

The analysis of variance of the Ea data obtained for glycosidases of P. fluorescens 26 indicated that there were significant differences among each of these enzymes. Duncan's Multiple Range test showed that the four Ea values differed from each other (P<0.05). β-D-galactosidase had the highest mean Ea, while N-acetyl-β-D-glucosaminidase had the lowest. The lower the Ea, the higher the relative activity for any enzyme. This does not mean necessarily that the enzyme with the lowest Ea will be the most active at any temperature. Since the velocity of a reaction is also affected by other factors, such as concentration of enzyme; concentration and availability of substrate; pH; ionic strength of the medium; temperature and presence of activators, catalysts or inhibitors; the Ea is only an approximate indicator of the activity of the enzyme at a given temperature (7).

Activity of glycosidases at 20°C

Another way of characterizing the glycosidases is to determine their activity at 20°C, the optimal temperature of growth for many psychrophilic and psychrotrophic bacteria (4). Table 2 gives units of activity at 20°C of the six glycosidases of P. fluorescens 26, the square root-transformed means and the results of Duncan’s Multiple Range test.

N-acetyl-β-D-galactosaminidase and β-D-galactosidase had the highest units of activity at 20°C. There were significant differences compared to the other glycosidases, but amounts of PNP released by these two glycosidases did not differ significantly. N-acetyl-β-D-glucosaminidase was the third highest in activity. β-D-glucoosidase, β-D-mannosidase and β-D-fucosidase, in this order, had the lowest activity with no significant differences among them.

Stability of glycosidases to pasteurization temperatures

All glycosidases were sensitive to pasteurization. Both heating processes, the low-temperature long-time (LTLT) and the high-temperature short-time (HTST), inactivated...
TABLE 3. Molar concentrations* of p-nitrophenol in minimal growth broth, inoculated with unheated and LTLT- and HTST-heated crude enzyme, determined after incubation (2 d at the optimal pH and temperatures of glycosidases).

<table>
<thead>
<tr>
<th>Glycosidases</th>
<th>p-nitrophenol concentration (x $10^{-10}$ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unheated</td>
</tr>
<tr>
<td>β-D-Fucosidase</td>
<td>1037</td>
</tr>
<tr>
<td>β-D-Mannosidase</td>
<td>551</td>
</tr>
<tr>
<td>β-D-Glucosidase</td>
<td>375</td>
</tr>
<tr>
<td>β-D-Galactosidase</td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-β-D-Glucosaminidase</td>
<td>53</td>
</tr>
<tr>
<td>N-Acetyl-β-D-Galactosaminidase</td>
<td>680</td>
</tr>
</tbody>
</table>

* Averages of duplicate determinations.

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REFERENCES


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