The Effects of Zinc on Chitinase Activity

An Honors Thesis (ID 499)

by

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ABSTRACT

Chitinase, an enzyme important in the degradation of chitin, is produced by the EF-4a bacterium. Heavy concentrations of zinc were found to inhibit enzyme activity. This decrease in activity was more prominent with the harvested crude enzyme than with the purified dialysate. One reason for this difference could be that chitin chelates the zinc, inhibiting its degradation and decreasing substrate availability. Because of lower protein concentration in the dialysate, excess chitin is present. This excess chitin is degraded by chitinase and products of enzyme activity remain constant. Another reason for the difference could be that a dual enzyme system is present in the crude. One of these enzymes is capable of degrading chitin which is chelated to zinc while the other enzyme cannot. The dialysate may contain only the enzyme that does degrade chitin which is chelated to zinc, having been purified through affinity chromatography. Therefore, the zinc does not interfere with degradation, and the chitin does react with the chitinase. Enzyme activity remains high and does not decline.

INTRODUCTION

With current interest in the use of chitin in medicine, agriculture, and industry, the chitin-chitinase enzyme system is
of great importance (1). Chitin is a polymer of B-1,4-N-acetyl-D-glucosamine and is widely found in the aquatic environment. Here it is important as it provides carbon and nitrogen for nutrient regeneration (9).

The purpose of this study was to determine the effects of different concentrations of zinc on the activity of a chitinase enzyme system. Various enzyme fractions of supernates of cultures of EF-4a were to be analyzed and compared with regards to incubation with zinc. Both soluble products of hydrolysis and identification of products by HPLC were to be performed.

Although several microorganisms have been found to be chitinolytic, the organism used in this study, the EF-4a, has not been widely used in research. Serratia and Vibrio spp. have often been studied for chitinolytic activity (17, 12).

Zinc is a heavy metal that is found naturally in the aquatic environment. In amounts greater than 5 mg/1 it is considered to be toxic (5). Since heavy metals are known toxic pollutants, their presence in the aquatic environment is a major concern (7).

Cell numbers or growth rates have often been used to measure toxic effects (3, 13). Unfortunately, these methods require long experimental times. Measurements of the metabolic activity of organisms give a more immediate indication of the toxic effects of heavy metals (2, 6).

MATERIALS AND METHODS

ORGANISMS. A culture of chitinolytic EF-4a was used for this study. It was grown on chitin agar made according to Skerman (15).

MEDIA. Chitin substrate was prepared by a modified method of
Molano, et. al. (8). Chitosan was acetylated with acetic anhydride in methanol, and the chitin was dialyzed to neutrality.

The cells were grown up in a chitin broth (Table 1) made up according to Smucker (16). Cultured supernates were harvested by centrifugation and concentrated by ultrafiltration using a YM 10 filter with 10,000 MW cutoff. Affinity chromatography was performed on concentrated fractions according to the methods of Roberts and Cabib (14).

PROTEIN ASSAYS. Protein determination was made by the Bio-Rad dye-binding assay described by Bradford (4). Absorbance was read on a Sequoia-Turner Model 320 Spectrophotometer at 595 nm. Protein was also checked by UV absorption at 280 nm using a Perkin-Elmer dual beam spectrophotometer (model 124).

CHITINASE ASSAY. Chitinase activity was determined by monitoring release of $^3$H-soluble products from $^3$H-labeled chitin. This method used was a modification of Molano, et. al. (8). Each reaction mixture contained 20 ul of $^3$H-collodial chitin, .5 ml of enzyme solution, and .25 ml of ZnSO$_4$ solutions of concentrations ranging from 0.0008 mg Zn/ml to 0.32 mg Zn/ml. The ZnSO$_4$ solutions were made up fresh each time the assay was performed. The mixture was incubated at 37$^\circ$ C for one hour, and the reaction stopped with .2 ml of 10% TCA. The mixture was filtered and collected in scintillation vials containing 9 ml of scintillation fluid. The Beckman model LS 3801 scintillation counter was used for counting.

HPLC. An attempt was made to show product variation with the use of HPLC. However, because of instrument malfunctions, this
was not accomplished.

RESULTS

Colorimetric protein determinations were performed on both the crude supernate and the dialysate of affinity chromatography. Concentrations of protein standards (γ-globulin) were plotted and used each time protein was analyzed. Unknowns were then read from this curve. Crude and dialysate concentrations are shown in Table 2 along with UV absorptions.

The chitinase assay was performed in duplicate on both the crude and dialysate. A distinct decrease in enzyme activity was seen with the crude, but little or none was seen with the dialysate. The sudden drop in activity was seen between 0.008 mg Zn/ml and 0.0162 mg Zn/ml. The zinc concentrations were plotted against CPK's (Figures 1-4).

DISCUSSION

There are several possibilities for the difference in results from the crude and purified dialysate. One takes into consideration that chitin is a chelating agent. The chitin pulls the zinc out of solution and binds to it. When this happens, the chitin may be incapable of reacting with the chitinase enzyme. Therefore, enzyme activity decreases. When the crude enzyme is reacted with chitin in the presence of zinc, there is a limited amount of chitin available. When small concentrations of zinc are used, not enough chitin is bound up to cause a decrease in enzyme activity. However, as zinc concentrations are increased, more chitin is required to bind with zinc, and sub-
strate availability decreases.

When the dialysate is reacted with chitin in the presence of zinc, there may be an excess of chitin present. This is because protein levels are lower in the dialysate. As zinc concentrations are increased, more chitin is used to chelate to the zinc. Enzyme activity does not decrease, however, because there is excess chitin available to react with the chitinase.

Another possibility for the difference in results is a dual enzyme system. Two enzymes could be present in the crude, either a chitinase and a chitobiase or two chitinase enzymes. One of these enzymes is capable of degrading chitin which is chelated to zinc while the other is not. The crude, having not been purified, contains both enzymes. When the crude is reacted with chitin in the presence of small concentrations of zinc, the zinc is chelated by the chitin, but enough chitin remains for degradation by one of the chitinases. Enzyme activity remains high. With increasing concentrations of zinc, however, more chitin is needed for chelation and less is available for degradation. Enzyme activity will then drop.

The purified dialysate, having been purified by affinity chromatography, contains only one chitinase enzyme. The enzyme it contains is the one capable of degrading chitin which is chelated to zinc. When the dialysate is reacted with chitin in the presence of zinc, the chitin is degraded by chitinase with no interference from zinc. This causes enzyme activity to remain stable even when high concentrations of zinc are added.

The level at which chitinase activity was found to decrease
in the crude was at 0.008 mg Zn/l or 8 mg/l. This is above the concentration designated as toxic or 5 mg/l (5). It would seem that the addition of zinc resulting in concentrations greater than 3 mg/l into the aquatic environment could greatly upset the balance of this environment. Because chitin could be chelated with zinc, the ability of chitin to be degraded biologically (enzymatically) would be diminished. Less carbon and nitrogen would then be provided for nutrient regeneration. This could then result in a subsequent loss of food in the environment.

Emphasis has focused thus far on substrate modification due to chelation. With known chelating ability of chitin, this would seem most likely (10, 11). However, another aspect yet to be examined is direct interaction of zinc on the enzyme(s) in question. This has not yet been resolved. Future experimentation should be directed at resolving this issue.
Table 1. Ingredients and amounts per 1 liter of Smucker's chitin broth

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>MgSO₄ • H₂O</td>
<td>1 g</td>
</tr>
<tr>
<td>FeSO₄ • 7H₂O</td>
<td>10 mg</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>300 mg</td>
</tr>
<tr>
<td>ZnSO₄ • 7H₂O</td>
<td>1 mg</td>
</tr>
<tr>
<td>MnSO₄ • H₂O</td>
<td>10 mg</td>
</tr>
<tr>
<td>CuSO₄ • 5H₂O</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>CoSO₄ • 5H₂O</td>
<td>0.01 mg</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>134 mg</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5 mg</td>
</tr>
<tr>
<td>CHITIN</td>
<td>1 g</td>
</tr>
<tr>
<td>YEAST EXTRACT</td>
<td>10 mg</td>
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</table>
Table 2. Protein concentrations and UV absorption (280 nm) for crude supernate and purified dialysate of EF-4a.

<table>
<thead>
<tr>
<th></th>
<th>PROTEIN CONCENTRATIONS</th>
<th>UV ABSORPTION (280)</th>
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<tbody>
<tr>
<td>CRUDE</td>
<td>20 ug/ml</td>
<td>.46</td>
</tr>
<tr>
<td>DIALYSATE</td>
<td>18 ug/ml</td>
<td>.13</td>
</tr>
</tbody>
</table>
Figure 1. Chitinase activity of crude enzyme fraction of ET-4a following exposure to zinc (Trial 1). The reaction mixture containing .5 ml enzyme preparation, 20 ul chitin, and .25 ml \( \text{ZnSO}_4 \) (0.0008 - 0.32 mg Zn/ml) was incubated for 1 hour at 37°C. Filterable products of \( ^3\text{H}-\text{chitin} \) were determined after termination of the reaction with 10% TCA.
Figure 2. Chitinase activity of crude enzyme fraction of EF-4a following exposure to zinc (Trial 2). The reaction mixture containing .5 ml enzyme preparation, 20 ul chitin, and .25 ml ZnSO₄ (0.0008 - 0.32 mg Zn/ml) was incubated for 1 hour at 37°C. Filterable products of ³H-chitin were determined after termination of the reaction with 10% TCA.
Figure 3. Chitinase activity of purified dialysate of BE-4a following exposure to zinc (Trial 1). The reaction mixture containing 0.5 ml enzyme preparation, 20 ul chitin, and 0.25 ml ZnSO₄ (0.008 - 0.32 mg Zn/ml) was incubated for 1 hour at 37°C. Filterable products of ³H-chitin were determined after termination of the reaction with 10% TCA.
Figure 4. Chitinase activity of purified dialysate of EF-4a following exposure to zinc (Trial 2). The reaction mixture containing .5 ml enzyme preparation, 20 ul chitin, and .25 ml ZnSO$_4$ (0.0008 - 0.32 mg Zn/ml) was incubated for 1 hour at 37°C. Filterable products of $^3$H-chitin were determined after termination of the reaction with 10% TCA.
COUNTS PER MINUTE ($x \times 10^3$)

LOG CONC. OF ZINC (mg/ml)


