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RESEARCH NOTES

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A MODEL SYSTEM FOR TESTING THE IMPACT OF TOXIC SUBSTANCES ON CHITIN DEGRADATION

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How can the ecological effects of a pollutant be measured since any ecosystem holds organisms of differing metabolic requirements and sensitivities? One method is to find a metabolic pathway common to organisms representative of many taxa in the ecosystem and to test the toxicity of the pollutant on that pathway. The biodegradation of chitin is one such pathway. Chitin is ubiquitous in any estuarine environment because it is structurally incorporated into so many types of organisms. It is a major component of shelly material in the Mollusca and Arthropoda, and is a constituent of fungal walls and of spores in the bacterium Streptomyces griseus. All chitin-containing organisms share a common metabolic requirement--they must be able to degrade chitin extracellularly before assimilating it. And every organism degrades chitin in the same way, by splitting or hydrolyzing the beta 1-4 linkage of the macropolymer and thus breaking it into smaller pieces. This extracelluar enzymatic hydrolysis of chitin is the rate limiting step of the complex metabolic pathway that results in the incorporation of chitin end-products into body tissues.

One difficulty in testing the toxicity of a pollutant to chitin degradation is that not all chitin is the same--it varies in its number of free or deacetylated amino groups. And as the chitin varies, so does its rate of enzymatic hydrolysis. What is needed to allow the comparison of toxicities to chitin degradation is a reference system, one whose only variable is the object of study--the presumed pollutant. In this paper the authors report a strategy for making a reproducible chitin--one with a constant degree of acetylation, for validating the ecological recognizability of the regenerated chitin and testing the toxicity of copper to its extracelluar degradation by the chitinase producing bacterium S. griseus. The method and materials described herein satisfy the requirements of a reference system.

An important concept underlying this work is that the biodegradation of chitin to its metabolically useful products is of greater ecological importance than its complete conversion to CO_2, the traditional consideration in biodegradation studies.

--The Editors

INTRODUCTION

As traditionally conceived, the biodegradation of a substrate is equated with its mineralization, or conversion to CO_2. We take as our premise the greater ecological relevance of considering the conversion of a metabolically unusable high molecular-weight polymer to its usable hydrolysis end-products. Okutani (1978) demonstrated that in the open ocean 100% of isolated organisms could transport and use the

hydrolysis end-products of N-acetyl-D-glucosamine (chitin) whereas only 10% could hydrolyze it. Given that 10^{11} metric tons of chitin are annually produced, its biodegradation is clearly an ecologically important metabolic task. Perturbation to this process is evidence of pollutant toxicity.

In this paper we develop a strategy for assessing the relative toxicities of water-borne pollutants to chitin biodegradation by measuring extracelluar enzyme (chitinase) activity, an indicator, we will demonstrate, of whole organism growth. Extracelluar enzymatic hydrolysis of chitin is the rate-limiting step in the metabolic pathway that results in incorporation of chitin hydrolysis end-products. Inasmuch as natural chitin varies in its degree of acetylation, and hence enzyme specificity, we utilize a method for preparing the substrate with a consistent degree of acetylation and reproducible particle size. We then demonstrate the acceptability of the synthetic chitin to naturally occurring organisms and test copper toxicity to Streptomyces griseus chitinase activity.

METHODS

Chitin Regeneration. Tritium (^3H) labeled chitin was prepared by a modification of the method of Molano et al. (1977). Chitosan, approximately 60% acetylated, was reacetylated with 25mCi ^3H-acetic anhydride in methanol/acetic acid. The resultant product was washed in a continuous-flow dialysis apparatus of our design. Each µl of prepared ^3H-chitin substrate contained 5.6 µg chitin.

Organisms. We selected S. griseus (Ohio State University Culture collection) as the reference for the toxicity assay because it produces the chitinase often sold industrially and is well established in the literature. Penicillium islandicum (ATCC) is of interest because it produces a chitosanase and thus will grow on chitosan but not chitin. Chitinolytic soil bacteria were obtained from Solomons Island, Maryland. The oyster, Crassostrea virginica, and comb jelly, Mnemiopsis, were obtained from the lower Patuxent River estuary. Microorganism-laden sediment was obtained from Flag Pond Bar, Chesapeake Bay, and Hungerford Creek of the lower Patuxent River. Water samples were obtained from Hog Island (Patuxent River) and from the Sargasso Sea.

Growth Media Preparation. ^3H-chitosan and ^3H-chitin were suspended in a Tris buffer medium of the following composition:

$MgSO_4.7H_2O$	0.1%
$FeSO_4.7H_2O$	0.001%
$ZnSO_4.7H_2O$	0.0001%
$CaCl_2O$	0.03%
$MnSO_4.H_2O$	0.001%
NaCl	1.0%
$CuSO_4.5H_2O$	0.000 005%
$CoSO_4.5H_2O$	0.000 005%
yeast extract	0.01%
K_2PO_4	134.0 mg L^{-1}
Tris HCl	1.39 g L^{-1}
Tris base	6.06 g L^{-1}

pH should be 7.6 without adjustment.

Inasmuch as either chitosan or regenerated chitin served as the sole carbon and nitrogen source in the media, growth is an indication of their degradation.

Phosphate is the more common buffering system for chitin media (Ohtakara et al. 1979). We tested both phosphate and Tris buffered chitin media on soil organism (actinomycetes) growth, as measured by plate count, and S. griseus growth, as measured by extracellular chitinase production, and achieved substantially better results with the Tris system in both cases.

Enzyme Assay. Enzyme solutions were diluted to naturally occurring concentrations. Because the reaction products were rapidly taken up by cells, cells were removed by centrifugation. Ten to 30 µl of ^{3}H-chitin was added to initiate the reaction, which, after 60 minutes, was terminated by the addition of 0.2 ml of 10% trichoracetic acid. Water soluble ^{3}H-oligomers were collected and assessed by liquid scintillation counting.

RESULTS

Chitin Validation. P. islandicum produces a chitosanase that allows growth on chitosan but not chitin. We tested P. islandicum growth on the regenerated chitin and the chitosan from which it was formed. The organism grew readily on the latter but not at all on the former.

Copper(II) binds to free amino groups, producing a characteristic blue color that is dependent on the number of free amino groups. Acetylated amino groups bind copper to a much lesser degree. Chitosan developed a deep blue color whereas the regenerated chitin produced a barely perceptible color at 1600 ppm $CuCl_2$. Further corroboration for our selection of the Tris buffer was evident in the deeper blue color development in a commercially available chitin prepared by swelling the chitin in phosphoric acid.

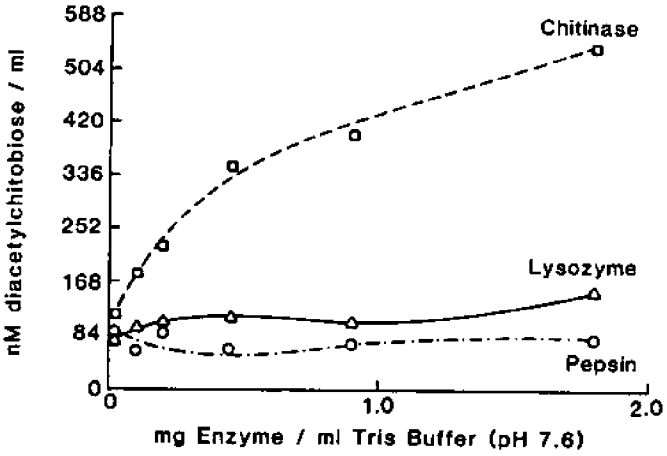

FIGURE 1. Specificity of ^{3}H-chitin substrate.

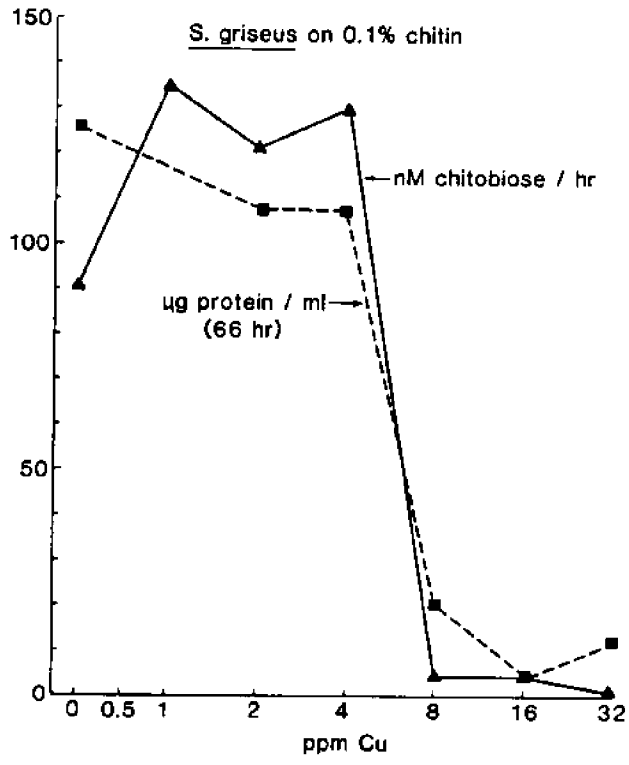

FIGURE 2. Mean cell-free enzyme activity (chitobiose/hr) and total protein production (protein/ml) of S. griseus grown on 0.1% regenerated chitin in Tris buffered medium.

So as to demonstrate that the regenerated chitin is specific to chitinase and is free of organic contaminants, and thus is the only available carbon and nitrogen source, we tested pepsin and lysozyme in addition to chitinase. The results, Figure 1, demonstrate that the regenerated chitin is acceptable to chitinase and is free of organic contaminants.

Of primary importance in judging the suitability of the regenerated chitin is its environmental availability, i.e., whether it is recognized and hydrolyzed by organisms in their environments. We tested extracts from oyster and comb jelly, and water and sediment samples from the lower Patuxent River estuary and the Sargasso Sea. We found chitinase activity in all samples tested except, as expected, the deep, biologically unproductive waters.

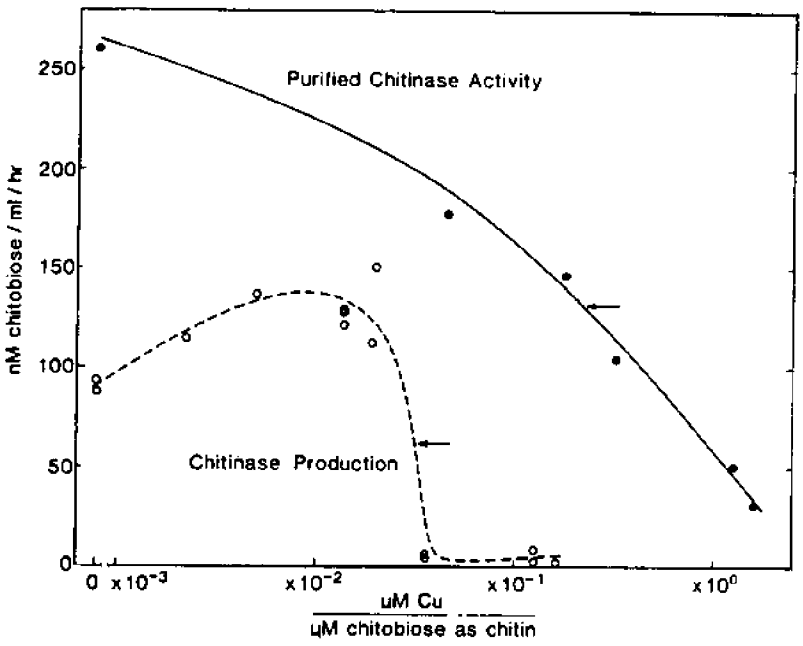

FIGURE 3. Normalized Cu^{+2} inhibition of S. griseus growth (chitinase production) and purified chitinase activity. Arrows indicate 50% reduction of control.

Copper effect on chitin hydrolysis. Copper toxicity was studied by enzyme assay and growth effect. The copper, presented as cupric chloride, was equilibrated with the chitin substrate. Then either enzyme was added, in the former case, or the medium was inoculated with S. griseus. The data presented in Figure 2, normalized to account for the different chitin concentrations in the two experiments (Figure 3), demonstrate that copper added in excess of 3-4 x 10^{-2} is inhibitory to enzyme activity, as measured by the number of chitin-degradative products released per milliliter per hour. That the inhibition in chitinase production reflects growth inhibition is shown in Figure 2; it is evident that enzyme activity parallels total protein production and thus growth. It is significant to note that for the whole organism chitinase production shown in Figure 3, copper was of beneficial effect in molar ratios less than 10^{-2}.

DISCUSSION

We have demonstrated the utility of considering toxic effect on chitinase as a measure of toxicity. The reference system proposed here, regenerated chitin and S. griseus, can be reproduced by other investigators considering other potentially toxic compounds so as to establish a scale of relative toxicities.

It is interesting to consider that the data in Figure 3 show a beneficial effect of copper at molar ratios of less than 10^{-2}. The regenerated chitin is more than 95% acetylated, i.e., 5% of its chitobiose repeating units have free nitrogen groups. Free nitrogen groups have greater binding capacity than do acetylated groups. Copper did not show toxicity in the whole cell (S. griseus) assay until added in excess of the number of free nitrogen groups. This demonstrates the importance of using a reproducible chitin for toxicity comparisons: these data suggest that apparent toxicity and degree of acetylation are negatively correlated.

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