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Tryptophan Repression of Exocellular Protease in *Neurospora*¹

H. Drucker, Biology Department, Battelle Pacific Northwest Laboratories,
Richland, Washington. 99352

We have demonstrated the ability of *Neurospora crassa* strain 7⁴A to grow on a medium consisting of Vogel's mineral salts, bovine serum albumin (BSA) as principal carbon source, and low levels of sucrose with an induction of exocellular protease parallel to growth. The induction of protease was found to be repressible by substrate quantities of a wide variety of metabolites (sugars, amino acids, peptides). In these initial studies, vegetative growth was initiated with conidiospores of the organism. Due to the low cell titer, the low level of protease in early growth, and the possible interference by germination in the process, the mechanisms of induction and repression for early stages in exocellular protease biosynthesis could not be examined.

To circumvent this difficulty, we developed a system using high concentrations of log-phase cells that permitted examination of repressing substances. *N. crassa* was grown 12 hours on Vogel's minimal medium plus 1% sucrose, harvested and suspended in Vogel's salts for a 30-minute period of starvation. This treatment was followed by a dilution of the starved culture (1:1) with 2% BSA in minimal salts medium. At this point I must digress for a moment to point out an omission in the abstract which is most important (slide 1). On this slide we plotted time of incubation in the BSA-containing medium against the units of proteolytic activity expressed as P.U. per ml culture filtrate. If the cells are first transferred to BSA, no significant amount of exocellular protease is secreted into the medium. Protease production is dependent upon the addition of

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either N. crassa induced exocellular protease or addition of the bacterial protease thermolysin in the same amount. In this case, and in all the work we shall discuss here, 0.080 units/ml culture of thermolysin were employed.

The mechanism of induction is beyond the scope of this presentation. However, it is necessary that I provide some background from our early work. This induction is not caused by hydrolysis of substrate by the added protease, but rather by the action of the enzyme on an inactive cell-bound protease in the presence of substrate, followed by two phases of induction. The first rate process shown here is dependent upon the units of enzyme added to the cell suspension and requires protein synthesis as it is completely inhibited by cycloheximide. The second rate process is also inhibited by the antibiotic. This rate, however, is not dependent upon concentration of added protease, but the appearance of this phase is dependent upon the first protease-dependent induction of exocellular enzyme. Please bear in mind the biphasic nature of this enzyme-dependent induction, as it will be of concern in our discussion of repressors.

As we had found that mixtures of amino acids were the most efficient repressors of protease biosynthesis in our early work, we examined the effects of low concentrations of individual amino acids on protease induction. These compounds were added to the BSA medium prior to the addition of log-phase cells and were present at concentrations between .98-.098 $\mu\text{m}/\text{ml}$. The dicarboxylic amino acids and their amines (that is, aspartic and glutamic acids) are without effect; and induction proceeds as shown on this first slide. This is also the case for the alcoholic amino acids (serine and threonine), proline, glycine, and cystine. The chain hydrocarbon amino acids, with the unexpected exception of isoleucine, have a marked effect on protease induction (slide 2).

I must apologize for the statement which you will find on the abstract that neutral amino acids are not repressors of protease. At the time the abstract was written our data were not complete. Note that the first process of induction is not a function of the concentration of these amino acids. The second process shows a decrease in rate that is dependent upon concentration of these amino acids. If we examine the percent change in rate of protease synthesis caused by all of these compounds, including methionine, as compared to a control to which no amino acids were added, we see that the effect of concentration on rate is similar for all these amino acids. That is, .98 $\mu\text{m}/\text{ml}$ alanine, valine and leucine cause a 43, 65, and 61 % decrease in rate, respectively. Methionine shows a change in the time for initiation of the second phase at this concentration, but not at the two lower concentrations, which is the reason for including it in this group of amino acids.

The basic amino acids, histidine, arginine, and lysine, show a quite different picture of repression from that of the neutral amino acids (Slide 3). In control cultures, the second rate process occurs at approximately 2.5 hours of incubation. This is also the case for cultures treated with hydrocarbon amino acids. Here, both the time of initiation for the second phase of induction and the rate of this phase are functions of the concentration of added amino acid. There is some similarity in the concentration dependence required for second phase of induction (neither lysine nor arginine have a second phase at 0.98 $\mu\text{m}/\text{ml}$) and, perhaps, some general similarity in the rate of second phase on concentration. This is not as clear as the data for chain hydrocarbon amino acids.

The aromatic amino acids have, perhaps, the most interesting effect on protease production (slide 4). The effect is on the time of initiation of the second phase of protease induction, but not on the rate of this process, with the qualified exception of phenylalanine. In the case of phenylalanine, the decrease in rate of the second process as concentration of amino acid increases is not very marked. Tryptophan, as you can see from this figure, is, in fact, the most potent of all amino acids in repressing protease biosynthesis.

We can divide all the amino acids tested into four groups, based on their repressing ability and, fortuitously enough, on their structural properties. Acidic and alcoholic amino acids proline, cysteine, glycine and isoleucine, have no effect on induction. The hydrocarbon amino acids (valine, leucine, alanine and methionine) affect rate of the second induction process only, which would be expected of a form of catabolite repression. The basic amino acid (lysine, histidine, and arginine) affect both initiation and rate of the second induction process. We might consider this a combination of catabolite repression, i.e., an effect on rate of gene transcription, and some relatively specific repression. We take this to mean that the amino acid prevents expression of the protease gene or genes, not the rate of such expression. i.e., message synthesis. We think that the action of the aromatic amino acids, and especially tryptophan, may be an example of this specific type of repression, as they do not affect the rate of the second induction process, but only the time of initiation of the process. As such, the aromatic acids may be unique effectors.

In any case, we have investigated the mechanism of tryptophan-mediated repression of protease biosynthesis. In *N. crassa*, excess intracellular tryptophan is converted to formyl kynurenine. This derivative of kynurenine

induces kynureninase activity in the cell, and these enzymes convert formyl kynurenine to formyl anthranilic acid which is secreted by the cell into the medium. We wished to see if tryptophan or its products were the agents of repression. The final product of tryptophan catabolism, formyl anthranilate, and the similar compounds anthranilate and 3-OH anthranilate had little or no effect on protease induction. The kynureninase inducing compounds (kynurenine, hydroxy-kynurenine, and formyl kynurenine) were identical to tryptophan in their repression of protease (slide 5). Again, these effectors affect the time of initiation of the second phase of protease biosynthesis, not the rate of the process.

This suggested to us that tryptophan repressed protease biosynthesis through the intermediate in its catabolism, kynurenine. If kynurenine is removed as anthranilic acid, the repression might be relaxed and induction might then occur. Evidence that this is the case was provided by a series of experiments similar to the one shown here (slide 6). Various concentrations of kynurenine were added to BSA-salts prior to the addition of log-phase cells. Exocellular protease and accumulation of anthranilic acid in the medium were determined. In this experiment, there is 0.098 $\mu\text{m}/\text{ml}$ of kynurenine in the medium. Anthranilate levels, determined fluorometrically, reached a constant value at 2 hours and the second phase of protease induction occurred normally; that is, at 2.5 hours of culture. As the kynurenine concentration is varied, definite correlations for time of initiation of the second phase of protease synthesis, rate of anthranilic acid secretion, and time at which anthranilic acid levels become constant can be seen (slide 7). Note that the rate of protease production (second phase) as pointed out before, does not vary with kynurenine concentration. Note that in all cases where protease was induced during the time course of the experiment, the time

of second phase followed the attainment of constant anthranilate levels in thirty minutes. The time it takes to reach the plateau level is obviously a function of the rate of anthranilate production (slide 8). Here we have the rate of anthranilate production against time of initiation for the second phase of induction. Note the correlation. The implication of these data is that formyl kynurenine may act as a repressor in protease induction. Removal of kynurenine from the cell as anthranilate by action of kynureninase relaxes the repression, allowing the normal second phase of induction to occur.

We have done the same experiment with tryptophan resulting in similar data. Since the abstract of this paper was submitted, we have found that kynureninase levels, using either tryptophan or kynurenine as inducer of kynurenine and repressor of protease synthesis, mirror the data for anthranilate production. This is what would be expected if kynurenine is the repressor in the tryptophan effect. We have found that levels of intracellular tryptophan, using tryptophan as a repressor in the protease induction, are consistent with the suggested scheme of regulation.

As Marshall McLuhan put it, "The Media is the Message". In the case of Neurospora, this appears to be so. The amino acid composition of a protein substrate employed to induce enzyme in a defined system as demonstrated here, or in an industrial fermentation, may affect the amount of enzyme made, the rate of synthesis of enzyme, or both. Cofactor sources, such as corn steep liquor, bran extract, and the like may vary in their effectiveness in proteolytic fermentation processes due to their amino acid composition. In the case of Neurospora, we hope to provide a full set of ground rules for maximal production and rate of production of proteolytic enzyme.

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