ENZYMATIC STUDIES OF RADIATION DAMAGE

Progress Report

M. Laskowski, Sr.

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Publications:

- C00-3225-25 Kowalski, D. and Laskowski, M., Sr., "Functional Characterization of Nucleophosphodiesterases", (in press, Handbook of Biochemistry, G.D. Fasman, ed., the Chemical Rubber Co., expected to appear March, 1976, no reprints are anticipated, six copies of the page proofs are enclosed).
- C00-3225-26 Kowalski, D. and Kroeker, W.D., "Physical, Chemical and Catalytic Properties of the Single-Strand Specific Nuclease from Mung Bean Sprouts", abstract (submitted, Fed. Proc.).
- C00-3225-27 Kroeker, W.D., Kowalski, D. and Laskowski, M., Sr., "Action of Single-Strand Specific Mung Bean Nuclease on Native DNA", abstract (submitted, Fed. Proc.).
- C00-3225-28 Perry, L.E., Pritchard, A.E. and Laskowski, M., Sr., "Preparation of Endonuclease, Exonuclease and 5'-Nucleotidase from the Same Sample of Venom", abstract (submitted, 5th International Symposium on Toxinology, Costa Rica, August, 1976).

Summary

A review on nucleolytic enzymes is expected to appear this month. Three abstracts were sent, two to Federation and one to the International Society on Toxinology. Further improvements were made in the method of preparation of venom exonuclease (phosphodiesterase). Other enzymes in venom hydrolyzing phosphate bonds were also partially purified. The major progress was made in the understanding of action of mung bean nuclease. Two papers not mentioned in publications were submitted to the Journal of Biological Chemistry and will be included in next year's report. The enzyme protein was characterized and the mechanism of action on native DNA was studied. Conditions were found to produce large (about 1 million dalton) duplex fragments from native T₇ DNA. Under different conditions, these fragments are degraded from their ends with the production of mono- to trinucleotides.

Research

In discussing last year's research, I stated that most of our effort was directed toward elucidation of the mechanism of action of mung bean nuclease, but no publishable results were obtained. This year, documents C00-3225-25 and C00-3225-26 are abstracts which will appear in the Federation Proceedings. The full size papers were also written and submitted to the Journal of Biological Chemistry. Only the abstracts are listed among this year's publications, the papers will be hopefully included in the next year's report.

The work on mung bean nuclease went in several directions. First, we succeeded in considerably simplifying the method of preparation of this enzyme, without losing the quality. The final preparations by the old and the new methods are as far as we can tell identical and fulfill the same criteria of homogeneity. The first two steps of the previous method (document C00-3225-3) remained unchanged except that the original frozen sprouted beans were not washed with water, but directly ground in a Waring blender with hot (55°) water. The elimination of washing the sprouts resulted in almost doubling the yield of the crude ethanol powder. As before, ethanol powder was accumulated from several preparations and was kept for many months without a noticeable loss of activity. From there on, the method differed. The new method consisted of 1) a heat step, 15 min, at 70°, pH 5.0 in presence of 1 mM Zn^{++} and 2 mM cysteine. The denatured contaminating protein was filtered off. 2) Ammonium sulfate fractionation between 50% and 80% saturation. 3) Chromatography

on DE-52 cellulose in 0.05 M sodium acetate, pH 6.0, 1 mM cysteine, 0.1 mM zinc acetate, with 0 to 0.35 M gradient in NaCl. 4) Chromatography on CM cellulose in 0.01 M sodium acetate, pH 5.0 containing 1 mM cysteine and 0.1 mM zinc acetate with a gradient in NaCl from 0 to 0.15 M. 5) Gel sieving on Sephadex G-100. The active peak shows a constant specific activity across the peak.

The simplified method, starting with ethanol powder can be finished within 4 days. The final product is homogeneous by several criteria and undistinguishable from that previously prepared. With the accelerated method it was possible to prepare sufficient amount of enzyme for general chemical characterization. The enzyme is a glycoprotein, it contains 29% carbohydrate by weight. It's molecular weight is 39,000 as determined by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate. The enzyme contains one sulfhydryl group and three disulfide bonds per molecule. The complete amino acid analysis was performed. The enzyme is rich in aromatic amino acids (12.6 mole %).

Under very carefully controlled electrophoresis conditions, the enzyme separates into two bands which are equally active. The first band, accounting for about 30% of the material, when reduced and treated with dodecyl sulfate, gives a single band around 39,000 daltons. The second band accounting for about 70% of the material, when reduced and treated with sodium dodecyl sulfate, gives two bands corresponding to 25,000 and 15,000 daltons. Without reduction, the second band, when treated with sodium dodecyl sulfate, gives a single band around 39,000 daltons. These results suggests that 70% of the enzyme molecules are cleaved in

a single region of the polypeptide backbone, and that two peptide fragments are held by one (or more) S-S bond(s). Interestingly, the enzyme prepared by the previous method also shows similar proportions of cleaved and uncleaved molecules.

A series of experiments was then performed on denatured DNA in which ionic strength and pH was varied independently. The results showed that increasing the ionic strength shifts the optimal pH toward the acid side. Similar results were observed with native DNA. However, it became obvious that on the acid side of the spectrum the preference for single-stranded versus double-stranded DNA decreases. That lead to a practical consequence. In order to create conditions in which double-stranded structure will be maximally discriminated, high ionic strength and high pH (about 0.5 pH unit above the optimum) must be preserved.

The experiment was then performed using intact T₇ DNA with a large amount of enzyme, high salt, high pH, low temperature. Under these conditions native DNA is reluctantly hydrolyzed into a large duplex fragments of relatively uniform size of about 1 million daltons. The amount of acid soluble materials is about 2%. Diluting the reaction mixture three fold with water and changing temperature from 22° to 37° resulted in a rapid formation of acid soluble material without a significant decrease in the size of fragments.

It was then decided to study the reaction in more detail. The 1 million duplex fragments were first prepared and used as a substrate in the most favorable conditions. The reaction was fol-

lowed by gel filtration. It was obvious that only small molecular compounds were produced and no fragments of intermediate sizes. The small molecular compounds were then examined by the method of Tomlinson and Tener and were shown to be composed of mono- to trinucleotides. A control experiment in which denatured DNA was used instead of a duplex fragment showed the formation of products of intermediate size and little accumulation of mononucleotides. We interpreted the observed results as end-preferring (exophilic) activity of mung bean nuclease acting on native duplex fragments.

In essence, this end-preferring activity appears to be an extension of the preference for single-stranded structures. The unwinding of all types of DNA starts from the termini. It is conceivable that in the process of unwinding the extended single-stranded ends are formed - which are an ideal substrate for mung bean nuclease.

Besides the work on mung bean, two adjacent topics were studied. One was the nuclease from Alcaligenes faecalis. As previously mentioned, we suspected that this organism produces an extracellular enzyme that can cleave across from a nick (inflicted by DNase I), but cannot cleave undamaged double-stranded DNA. We postulated the existence of such an enzyme on the basis of suggestions and implications in the published literature. During the work, we soon found that the organism produces a number of nucleases, but none with the postulated properties. One of these nucleases appears to be an exonuclease and may be worthy of purification and characterization. I do not think, however, that the problem is sufficiently urgent and offering rewards justifying

immediate attention. As a result, we decided to postpone the work on the topic and "table it" for the time being.

Contrary to the judgement expressed in the last year's report, we have found that the problem of venom exonuclease (phosphodiesterase) is not solved once and for all. Repetition of our previous method showed that the preparation is about 95% pure, and two, very faint bands can be detected by gel electrophoresis. This finding reopened the study of exonuclease and document C00-3225-28 is the abstract concerned with this problem. In the previous method, step 4 was chromatography on QAE-Sephadex. During this step, the enzyme came out in a sharp peak with the front, and only a small amount of protein of low activity was eluted after a gradient had been applied. We were able now to substitute a different step based on affinity chromatography, mainly an NADP-column. The column has been described by Dr. Oleson who was kind enough to send us a preprint prior to publication in Analytical Biochemistry. Substituting NADP-column for QAE-Sepharose resulted not only in an improved purification step, but also in an improved reproducibility.

The return to the study of exonuclease also wetted our appetite for better utilization of venom. In the previously reported method, document C00-3225-16, prior to the isolation of exonuclease, venom was incubated at pH 3.6 at 37° for 3 hr to destroy most of the 5'-nucleotidase. This last enzyme is not insignificant, during the past year I received three calls asking for a partially purified 5'-nucleotidase which we made some 15 years ago. It started the following trend of thought: If step 1 in the preparation is de-

vised to grossly separate exonuclease from 5'-nucleotidase, it may be possible to obtain both enzymes from the same sample of venom, thus cutting the investment in very expensive source of these enzymes. The preliminary results are sufficiently encouraging not to disregard this alternative. Upon separation, exonuclease can be freed from the remaining 5'-nucleotidase by exposure to acid. Document C00-3225-28 describes the procedure based on this order of steps using NADP affinity chromatography as a last step.

The possible purification of contaminating 5'-nucleotidase, and incidentally also of venom endonuclease, was looked at. Since all three enzymes are glycoproteins of high molecular weight, affinity chromatography on NADP based on absorption at optimal pH value of enzyme activity offers a method with the potential of separating exo- from endonuclease.

Nuclease from salmon testes has been applied to digest crab d(AT)_n polymer. So far, the results are not only far from publishable, but even difficult to discuss in the report. It is hoped, however, that in the next year's report it may become possible. The general aim is to explain the presence of a small amount of RNA, which is highly resistant to a combination of pancreatic RNase and T₁ RNase. Two alternatives are considered: 1) That RNA is covalently incorporated and 2) that fragments of RNA are perfectly matched to DNA and thus made inaccessible to RNases.

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NAMES, DEPARTMENT, AND OFFICIAL TITLES OF PRINCIPAL INVESTIGATORS AND OTHER PROFESSIONAL SCIENTIFIC PERSONNEL: (not including graduate students) engaged on the project, and fraction of man-year devoted to the project by each person.

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SUMMARY OF PROPOSED WORK: (200-300 words, omit Confidential Data). Summaries are exchanged with government and private agencies supporting research, are supplied to investigators upon request, and may be published in AEC documents. Make summaries substantive, giving initially and for each annual revision the following: OBJECTIVE; SCIENTIFIC BACKGROUND FOR STUDY; PROPOSED PROCEDURE; TEST OBJECTS AND AGENTS.

Continuation of the characterization of mung bean nuclease, particularly with respect to the initial cleavages of native DNA. Study of large fragments formed with respect to termination.

Modification of the purification procedure of venom exonuclease aiming at simultaneous purification of 5'-nucleotidase and venom endonuclease.

Application of DNases to the study of composition of light satellite DNA from crab Cancer borealis.

RESULTS TO DATE:

Characterization of mung bean nuclease as a glycoprotein with one internal peptide bond cleaved and demonstrating the end-preferring (exophilic) activity of this enzyme.

	PROGRAM CATEGORY NO.
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M. Laskowski
Signature of Principal Investigator

DATE: March 11, 1976

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