To the Graduate Council:

I am submitting herewith a dissertation written by Gerald Edwin Spady entitled "The Intracellular Proteases of Neurospora crassa." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Frank H. Gaertner, Major Professor

We have read this dissertation
and recommend its acceptance:

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THE INTRACELLULAR PROTEASES OF NEUROSPORA CRASSA

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Gerald Edwin Spady

December 1980

to my parents

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In any venture encompassing a number of years and extended effort, one is encouraged and aided, if fortunate, by several others. I was so encouraged and aided, and sometimes dragged, by the following individuals to whom I express my appreciation: Ken Cole, who survived lightning, Frank, Tony, and myself and remains one of the most remarkable individuals it's ever been my pleasure to have coffee breaks with; Bonita Elmore, who drew all the figures, kept her sanity, and revealed herself as a warm and funny human bean; Barbara Moorman, who always managed to cheer me up on her way to the ice machine; Ed Hiss, who still believes in Notre Dame and the tooth fairy; Marge Horne, my verbal sparring partner, who is destined to reincarnate as an acorn; John Gresham and Sam Simmons, master craftsmen who taught me more than they perhaps know; Alonzo Standifer, who always held forth with thoughtful and enlightening discussion, and taught me to make a decent hamburger; Robin Rothrock, the Cornbread Fairy, who became a welcome member of the lunch bunch; Bob Wykle, who shares my love for fine wood and donated the truck used to get more; Anthony Vitto, Jr., who gets curiouser and curiouser and acknowledged me in his dissertation; Bob Horne, who ranks high on my list of good people to hang around with and whose advice, when requested, always seems to make sense; Don Foard, who could probably inspire another Italian Renaissance if given the opportunity; Charley and Barbara Mead, who show all of us how to live fully; Bob Cumming, who probably dislikes meetings as much as I do; Ed Phares, who grows good Neurospora and knows Corvairs; Lee Shugart, who grows good tomatoes and guided me as a committee member; Tom Slaga,

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ABSTRACT

A series of survey experiments designed to detect endogenous proteolytic activity in Neurospora crassa is described. The assay used azocoll, a highly sensitive, economical, general substrate. The disadvantage of azocoll's aqueous insolubility was overcome through design and manufacture of a solid-reagent dispensing machine, which allowed azocoll to be aliquoted precisely, quickly, and efficiently.

Lyopilized mycelia were fractionated three times with ammonium sulfate and chromatographed on diethylaminoethyl cellulose. The chromatographic fractions were then assayed for the presence of acidic, neutral, and alkaline proteolytic activities. Multiple proteolytic activities were demonstrated, based on the criteria of chromatographic location, pH optimum, inhibition by commercial and/or endogenous inhibitors, location in a particular ammonium sulfate preparation, growth stage of organism, and crypticity. Cryptic behavior was expressed by incubating column fractions at 4°C for several days and periodically assaying for the presence of proteolytic activities.

Substrate-specific peptidases were revealed by electrophoresing column fractions on 7% polyacrylamide slab gels and then incubating the gels in the presence of a peptide/dye solution, which displayed peptide digestion as sharp blue bands. Eighteen distinct peptidases were shown with this assay. It is concluded that N. crassa likely contains many more endogenous proteases than heretofore described for this, or any other, organism. The finding warrents further investigation to determine the number, location, and function of the endogenous multiple proteolytic activities.

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF FIGURES

FIGURE PAGE

LIST OF ABBREVIATIONS

ala	alanine
asp. acid	aspartic acid
DEAE	diethylaminoethyl
gly	glycine
his	histidine
leu	leucine
met	methionine
PCMB	parachloromercurobenzoate
phe	phenylalanine
PMSF	phenylmethylsulfonylfluoride
poly arg	polyarginine
pro	proline
TCA	trichloroacetic acid
tyr	tyrosine
val	valine

Beware the man who works hard to learn something, learns it, and finds himself no wiser than before.

He is full of murderous resentment of people who are ignorant without coming by their ignorance the hard way.

The Books of Bokonon

CHAPTER I

GENERAL INTRODUCTION

Dissertation Objectives and Format

The original objective of this dissertation was to isolate the five Neurospora crassa proteases reported by Siepen, Yu and Kula (46). The purified proteases were to be investigated for their effects upon Neurospora's arom conjugate, a single polypeptide containing five enzymes involved in tryptophan synthesis and known to be susceptible to proteolysis by endogenous proteases (21, 54). Initial experiments differed from Siepen et. al. only in the method of assay; the results suggested there were far more than five endogenous proteases in Neurospora. Accordingly, the objective was modified and a more rigorous investigation was begun with the goal of determining the number of endogenous proteases in Neurospora crassa.

The format of this dissertation was chosen to facilitate subsequent publishing of the data in technical journals. Chapters II-VI are organized as manuscripts, each containing a summary, introduction, materials and methods, results, and discussion. A final chapter has been added to discuss the implications of the findings. The single list of references at the end of the dissertation is applicable to all chapters.

Proteases: Importance and Classification

In any given cell, whether it represents the complete organism or only a minor component of a more complex organism, there are thousands of proteins. These proteins serve the cell and ultimately, the organism in a variety of ways, from providing a mechanical framework for cellular functions to being involved in nearly every chemical reaction taking place within the cell. When the need arises, cells manufacture proteins, oftentimes from raw materials such as a carbon and nitrogen source. This manufacturing process, or protein synthesis, has been the subject of much investigation and is now well understood (42).

Also in response to a changing environment, cells degrade the proteins they previously synthesized, as well as those proteins and peptides they obtain from their surroundings. This synthesis/degradation cycle of endogenous proteins is commonly called protein turnover (6, 43). The rate of turnover is usually expressed in terms of half-life and, for individual proteins, may vary from less than ten minutes to several days (17). Thus a given cell is actively both synthesizing and degrading protein. Indeed about 40% of rat liver protein is degraded daily (17). But whereas the mechanics of protein synthesis are clearly defined, the mechanics of protein degradation have not received as much attention, nor is this process well understood.

Protein degradation is accomplished by a particular class of enzymes called proteases (Enzyme Commission's sub-group 3.4) (18). Proteases may be divided into two large subclasses, depending upon

where the substrate protein is cleaved. If hydrolysis occurs on either end of the protein, the protease is termed an exopeptidase or peptidase. Furthermore, if the amino acid is removed from the amino terminus of the substrate protein, the peptidase is called an aminopeptidase, in contrast to a carboxypeptidase, which removes amino acids from the substrate's carboxyl terminus. The other subclass of proteases cleaves their substrate proteins at internal sites and are therefore called endopeptidases, or proteinases. Unique among enzymes, the endopeptidases are classified according to inhibition of their catalytic active centers, rather than through use of substrates as is normal in the identification of other enzymes. The endopeptidases are subdivided into serine-, thio-, carboxyl-, metallo-, and unclassified proteinases, depending upon the type of inhibition to which the proteinase is susceptible (6).

Proteases: A Brief, Historical Perspective

This summary is intended only to acquaint the reader with some of the salient discoveries in the research area of proteases. Much of the information given here is drawn from three excellent review articles, to which the reader is referred for a more thorough perspective (5, 22, 23).

Proteases in mammalian cells and tissues were first reported in 1890 by Salkowski, a German physiologist, who noted chemical changes in liver, muscle, and adrenal gland incubated for long periods at 37°C. Nearly 100 years prior to this, Vauquelin reported

proteolytic activity in plants (41). Jacoby introduced the term "autolysis" and in 1900 partially purified the enzyme which is now recognized as Cathepsin D. The term "cathepsin" was first proposed by Willstatter and Bamann to identify those acidic proteases found in a variety of tissues and now known to by lysosomal in origin (3, 33, 45).

From 1900 to the early 1920's came reports of proteases from such diverse sources as dog bone marrow, normal and pneumonic human lung, sputum, protozoa, bovine spleen, and bacteria. Late in this period the importance of pH became recognized, and proteases were classified according to their activity at acidic, neutral, and alkaline pH. During the period 1924-1935 four major proteases were investigated: trypsin, pepsin, papain, and the cathepsins. In 1930 Waldschmidt-Leitz isolated and identified, as glutathione, a naturally occurring activator of the cathepsins. The discovery set the stage for subsequent classification of proteases by inhibition of the catalytic mechanism of the enzyme's active site.

Prior to 1930 research involving proteases was hampered for lack of a suitable assay. Substrates at that point included tissue, fibrin and heated serum proteins, egg albumin, or gelatin. Cleaved products were precipitated by inefficient methods, and were then assayed for total nitrogen. In an attempt to provide more suitable assay conditions, Fruton and Bergmann prepared various synthetic substrates during the 1930's. Their efforts culminated in the synthesis of substrates specific for pepsin, trypsin, chymotrypsin, and papain. Shortly thereafter in 1940 Anson developed the hemoglobin

assay for endopeptidases, which is currently used as a general substrate assay.

In 1949 Linderstrom-Lang and Ottesen coined the term "limited proteolysis" to describe the transformation of ovalbumin to plakalbumin under the influence of a microbial protease, later known as subtilisn (34). This was quickly followed by the discovery that limited proteolysis was the primary chemical event in zymogen activation (15, 40). More recently, limited proteolysis has been broadened to encompass such diverse events as the initial step in protein catabolism (24), selective inactivation of sporulation enzymes (52), or assembly of yeast cytochrome c oxidase (37).

Despite the progress of the 1940-1950 era, most researchers regarded proteases as enzymes residing in the lysosomes or, analagously, plant vacuoles and mainly responsible for catabolism of cellular and extracellular bulk protein. This view was sustained because the research centered around the cathepsins, found primarily in the lysosomes, or the gut proteases trypsin, chymotrypsin, and pepsin. With the advent of new techniques, such as the use of radio-labeled substrate protein, more sophisticated chromatographic separation techniques, or increasingly more available and more specific artificial substrates, current research has unveiled much more subtle roles for proteases, particularly in the area of biochemical regulation of cellular functions. For instance we now know that proteases play a key role in: i) maturation of newly synthesized proteins (45), ii) selective activation or inactivation of enzymes (10, 11, 30), iii) the blood coagulation cascade (16), iv) degradation

of nonsense proteins (28), and v) development and differentiation(44). This list is merely representative, and by no means exhaustive.

The current view of the cellular function of proteases is often a curious mixture. Many researchers acknowledge the subtle intracellular modifications of which proteases are capable, while at the same time believing that the important functions of proteases remain those of bulk protein catabolism. This dissertation presents evidence that a multiplicity of intracellular proteases may require reanalysis of the possible functions proteases perform in regulation of cellular biochemistry.

CHAPTER II

A SOLID-REAGENT DISPENSER FOR USE IN THE AZOCOLL PROTEASE ASSAY (AND OTHER INSOLUBLE SUBSTRATE ANALYSES)

Summary

A machine designed to deliver accurate and repeatable quantities of an insoluble protease substrate, azocoll, is described. It makes feasible large-scale assays for protease with azocoll as a general substrate. The assay conditions described allow detection of trypsin in the nanogram range. The assay is compared with two other general methods.

Introduction

Azocoll has been used for many years as a general substrate for the assay of collagenases and other proteases (36). The assay is sensitive, simple to perform, and the reaction has essentially no background absorbance. Despite these advantages, azocoll has not been extensively used, primarily because the reagent is insoluble. This property normally requires the investigator to measure precise amounts of dry azocoll into each assay container. We have developed a solid-reagent dispenser to aliquot azocoll rapidly and accurately, and have used the machine to demonstrate multiple proteolytic activities in Neurospora crassa (20).

Materials and Methods

Azocoll-dispensing machine

The dispenser consists of three parts. The first part is a hopper, machined from a block of aluminum and containing 12 wells through which azocoll is funneled into the second part, a machined Teflon rod. This has 12 holes drilled into it, each containing space for approximately 18 mg of azocoll. It may be rotated 180° so that in one position (holes pointed up) all twelve holes are simultaneously filled with azocoll from the hopper, while in the second position (holes pointed down) the aliquots of azocoll are released from the rod and drop directly into test tubes positioned below the holes. The third part consists of an ordinary test tube rack with a Plexiglas template to hold each row of test tubes vertically and in position directly below the rod. Figure 2.1 is a photograph of the apparatus.

Figure 2.2 contains sectioned detailed drawings of the apparatus. The cross section of the turning knob (Section AA-1) shows the position of the positive lock pin, which allows the knob to be turned only 180° in either direction and thus aligns the holes in the rod with the hopper or with the test tubes. Section BB shows the hopper, the Teflon rod, and the Plexiglas template that houses the test tubes. The azocoll is fed directly into the hole in the rod; the rod is subsequently turned 180° and azocoll is deposited into the test tube. Section BB-2 gives the position of one of the positive lock pins which serves to align the template directly underneath the rod.

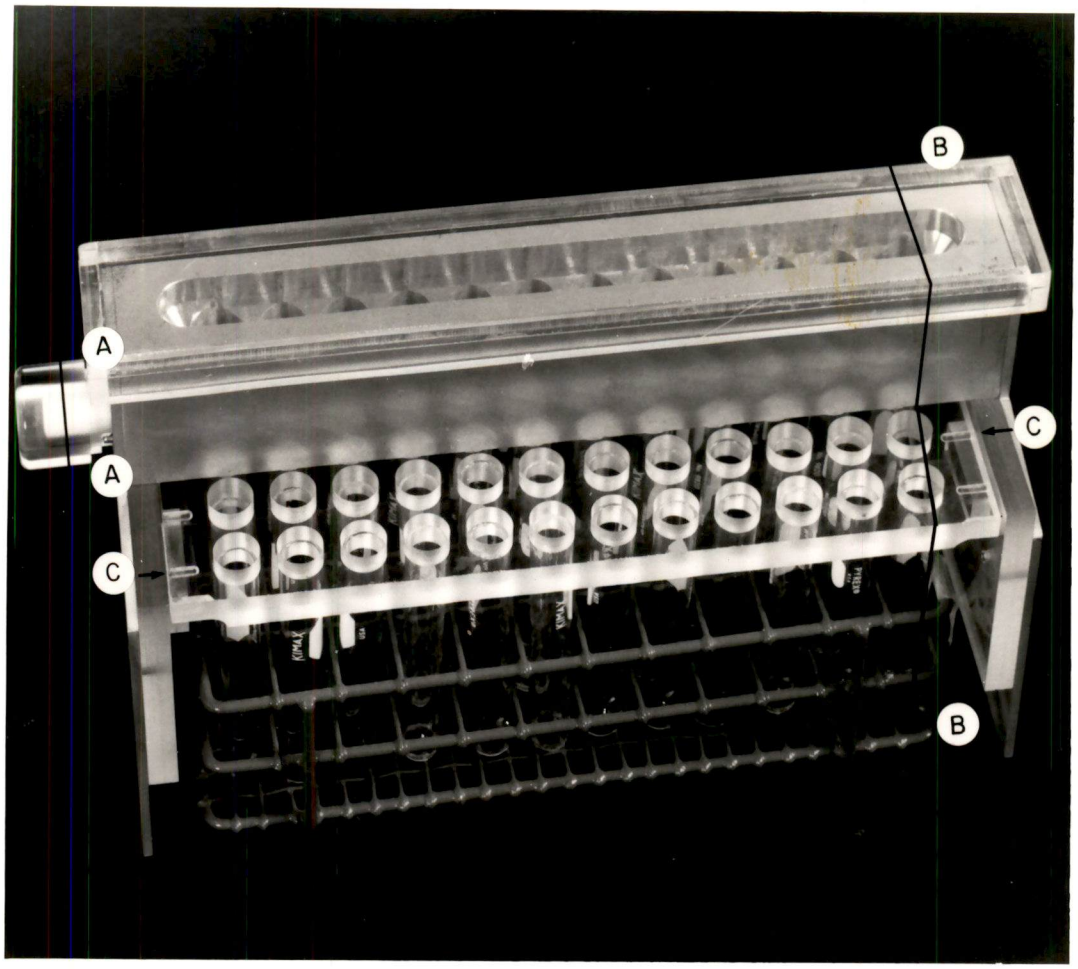

Fig. 2.1. Photograph of the solid-reagent dispenser.

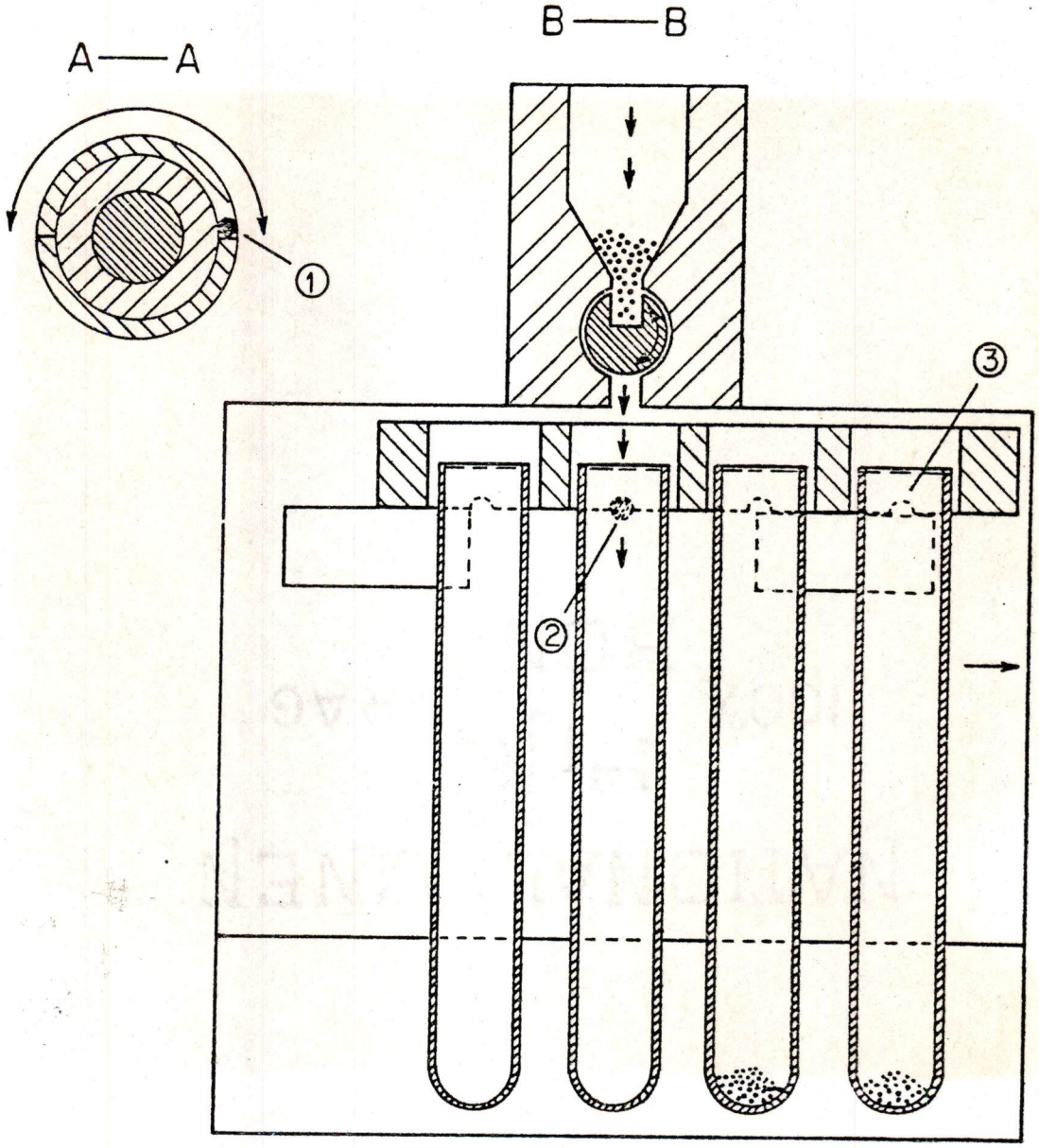

Fig. 2.2. Sectioned detail drawings of the solid-reagent dispenser.

Another lock pin is located on the left-hand side of the apparatus (not visible), and the corresponding stops in the template are shown in Fig. 2.1C, and in Section BB-3, Fig. 2.2.

The volume of the aliquot delivered is dependent solely upon the diameter of the rod and the diameter and depth of the hole drilled into it. Investigators would need to determine their own requirements, and construct accordingly. The overall size of the apparatus is determined principally by the size of the test tube rack and the test tubes that are used. We have not given any dimensions, but our rack holds 48 13 X 100 mm test tubes.

Azocoll assay method

The reaction mixture contained 18 mg of azocoll, 0.05 ml of enzyme sample (either from a column fraction or a known concentration of a commercial protease), and 0.45 ml of buffer. We used either 0.1 M potassium phosphate (pH 7.0 or 8.5) or 0.1 M sodium citrate/citric acid (pH 5.0), depending upon the protease investigated. The azocoll is dispensed into 13 X 100 mm test tubes, the buffer and enzyme are added to initiate the reaction, and the tubes are shaken with sufficient vigor to keep the azocoll in suspension. The standard reaction time is 10 hours. The reaction is terminated by centrifuging the test tubes at 2000 x g for 10 min to pellet the unsolubilized azocoll. The supernatant is aspirated into clean test tubes, and the absorbance is measured at 520 nm on a Model 2400 Gilford spectrophotometer.

Hemoglobin assay method

Tryptic digestion of hemoglobin was done according to a modified method of Kunitz (27). An 0.18 ml aliquot of a 1% hemoglobin solution (1 g denatured hemoglobin in 100 ml of 0.01 M potassium phosphate, pH 7.0) was measured into 10 X 75 mm test tubes. The reaction was initiated by the addition of 0.02 ml of enzyme sample. At designated time intervals 0.2 ml of 10% trichloroacetic acid was added to terminate the reaction. The samples were centrifuged at 2000 x g for 10 min, the supernatant was aspirated into clean test tubes, and the absorbance was measured at 280 nm.

Casein yellow assay method

Tryptic digestion of casein yellow was done according to a modified method of Anson (2). The procedure was the same as with hemoglobin except that a 1% solution of casein yellow was substituted for hemoglobin and the absorbance was measured at 422 nm. All assays were done at 25°C.

Materials

Azocoll and casein yellow were purchased from Calbiochem. Denatured hemoglobin and trypsin were purchased from Sigma. All other chemicals were reagent grade.

Results

We compared the sensitivity of the azocoll assay to detect tryptic hydrolysis with two other common protease assays using hemoglobin (27) and casein yellow (2) as substrates. The latter

assays are short-term, so we modified our procedure to include a reaction termination step, adding 0.5 ml of 10% trichloroacetic acid at appropriate time intervals. We found that azocoll is approximately 100-fold more sensitive, over a 30 min time interval, to detection of hydrolysis by trypsin than are either casein yellow or hemoglobin (Fig. 2.3).

Discussion

The solid-reagent dispenser greatly reduces the time needed to aliquot azocoll for each reaction mixture and makes feasible the use of azocoll for large-scale protease assay experiments. This method of measuring azocoll is both rapid and accurate (machine error is ± 4%). The dispenser could be used to measure other insoluble substrates or, by using Teflon rods with suitably-sized holes drilled in them, be modified to dispense different amounts of solid substrate.

Figure 2.3 shows that azocoll is indeed a more sensitive detector of trypsin hydrolysis than are either hemoglobin or casein yellow. Azocoll is also exceptionally stable (in the absence of protease activity) in water. This property insures a very low level of background activity, whereas the pink to red color that is released during proteolysis allows the investigator to visually scan the array of reaction tubes and quickly locate the presence of proteolytic activity in the samples assayed.

We found that, because of azocoll's stability, we could conviently run proteolytic assays over a longer period of time and

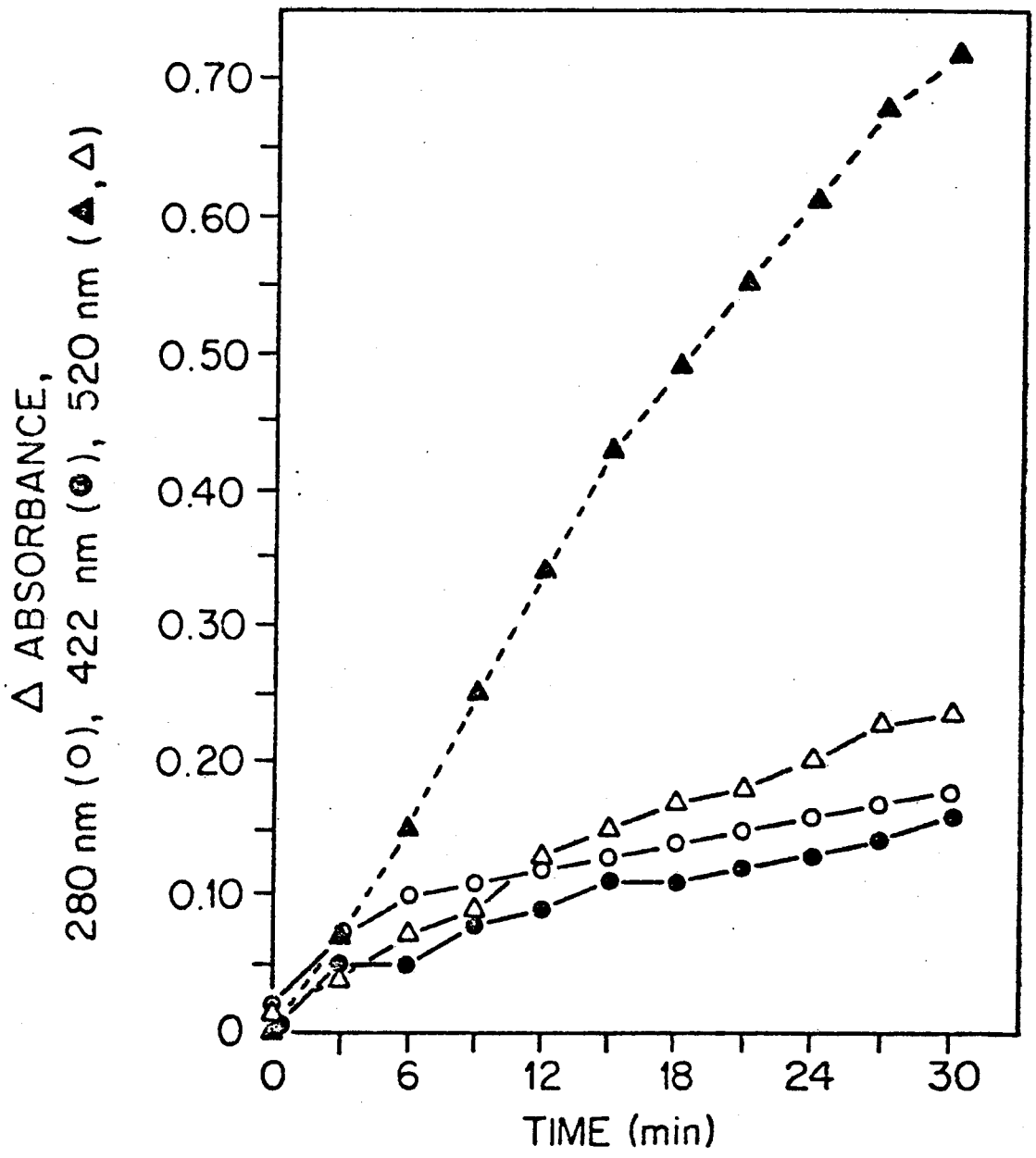

Fig. 2.3. Comparison of three short-term protease assays. Hemoglobin, casein yellow, and azocoll are compared for their ability to detect tryptic hydrolysis over a short time interval. The hemoglobin (●) and casein yellow (○) reaction mixtures each contained 1% substrate and 0.1% (1.0 mg/ml) trypsin. The azocoll reaction mixtures contained 18 mg of substrate and either 0.01 mg trypsin/ml (△) or 0.033 mg trypsin/ml (▲). Reactions were terminated by addition of 10% trichloroacetic acid.

thereby increase the assay's sensitivity to small amounts of protease. By using a ten-hour incubation period, we could readily detect one-nanogram quantities of trypsin, subtilisn, and thermolysin.

The solid-reagent dispenser solves the problem of aliquoting azocoll rapidly and accurately. We now use this substrate to assay large numbers of chromatographic samples for the presence of proteolytic activity, and we have found the assay to be sensitive, economical, and simple to perform.

CHAPTER III

EVIDENCE FOR A MULTIPLICITY OF PROTEASES IN *NEUROSPORA CRASSA*

Summary

Proteolytic contaminants cause an artificial subunit structure in the *arom* enzyme conjugate in *Neurospora crassa*. In an attempt to resolve this problem and to find conditions favorable for protease-free isolation of the *arom* conjugate, we examined the endogenous proteases of early logarithmic and stationary growth phases of *N. crassa*. Each stage was fractionated three times with ammonium sulfate and then separated by ion exchange chromatography. Each chromatographic fraction was assayed at acidic, neutral, and alkaline pH conditions with azocoll, a highly sensitive, general substrate. In addition, the chromatographic fractions were assayed in the presence of several natural inhibitors or commercial inhibitors to aid in defining the many proteolytic activities expressed in the presence of azocoll. Much of this activity was cryptic and was assayable only after preparations had been stored. We conclude that there are at least 26 distinct proteases in *N. crassa*. In addition, we consider it likely that there are many more than this number and that they are involved in a heretofore unrecognized complexity in the regulation of development and enzyme function.

Introduction

There is a growing appreciation of the diverse and important roles proteases play in cellular metabolism and physiological functions

within organisms. Proteases are involved in the well-known extracellular nutritive functions and in intracellular activities such as protein turnover, zymogen activation, and regulatory functions involving limited proteolysis (23, 55). They are also involved in intercellular roles such as production of blood clots and penetration of an egg cell by sperm during fertilization (23, 55). Perhaps equally important from a practical viewpoint is that proteolysis during protein purification procedures (21) can give rise to unsuspected artifacts.

Proteases have been extensively studied in microorganisms (55). Yeast contains four well-known proteases, while in Escherichia coli six of these degradative enzymes have been discovered (55). A report from our laboratory has described a proteolytic contaminant that was responsible for an artifactual subunit structure in the Neurospora crassa arom conjugate, a single polypeptide that catalyzes five sequential enzymatic activities in the central aromatic amino acid biosynthetic pathway (21). Siepen et. al. (46) reported the isolation of five separate proteases from N. crassa.

To deal with the protease problem (21) in N. crassa more effectively, we initiated a systematic study of the proteolytic activity involved in the in vitro degradation of the arom conjugate. We examined both stationary phase and early logarithmic cultures, hoping to find the most favorable growth conditions for isolation of the arom conjugate. Here we report the rather surprising result that far more than five proteases may be present in N. crassa and discuss some of the practical,

as well as some of the important physiological, implications of these findings.

Materials and Methods

Growth of organisms

N. crassa was grown as described earlier (19) and harvested at an early logarithmic growth stage (7 g/l wet weight) and stationary phase (21 g/l wet weight). The mycelia were lyophilzed, ground in a Wiley mill, and stored at -20°C.

Protein fractionation

Lyophilized mycelia (150 g) were mixed in 0.01 M potassium phosphate, pH 7.0, for 30 min and centrifuged at 10000 x g for 30 min. Nucleic acids were precipitated by stirring the supernatant with protamine sulfate (0.21% vol) for 15 min, and then centrifuging it at 10000 x g for 15 min. Fine crystals of ammonium sulfate were slowly added to the supernatant to 40% saturation (0-40% ammonium sulfate fraction), dissolved, mixed for 15 min, and centrifuged at 10000 x g for 15 min. In a similar fashion 40-50% and 50-80% ammonium sulfate fractions were prepared. The ammonium sulfate pellets were dissolved in a minimal volume of phosphate buffer. All preparations were done at 4°C. For purposes of comparison, the early log and stationary phase mycelia were fractionated in parallel.

Chromatography

Each ammonium sulfate fraction was placed on a 4 X 80 cm column of Sephadex G-25 equilibrated with 0.01 M potassium phosphate, pH

7.0, and the protein-containing eluant (volume~300ml) was pumped onto a 2 X 80 cm column of DEAE cellulose equilibrated with 0.01 M potassium phosphate, pH 7.0. The column was eluted with 0.01 to 0.25 M gradient of NaCl in 0.01 M potassium phosphate, pH 7.0. Fractions (12 ml each) were collected until the gradient was complete. One ml aliquots were removed from odd-numbered fractions and stored in a freezer at -20°C. The remainder of the fractions were stored in a freezer at -10°C. Additional aliquots were taken after six and ten months of storage.

Preparation of natural inhibitors

Stationary phase inhibitors were prepared by following the procedure outlined for protein fractionation with the following modifications: 10 g of lyophilized mycelia was the starting material, and the ammonium sulfate fractions were 0-40, 40-50, 50-60, 60-70, and 70-80%. One ml aliquots of the dissolved ammonium sulfate pellets were then boiled for five min and centrifuged for 15 min at 10000 x g. The supernatants, containing the heat-stable natural inhibitors, were diluted 1 to 1000 in the reaction mixture.

Azocoll assay

Chromatographic fractions were assayed for endogenous proteolytic activity using the general substrate, azocoll, as described earlier (47).

Chemicals

PCMB, PMSF, and benzamidine were purchased from Sigma. Azocoll and Trasylol were purchased from Calbiochem. DEAE cellulose No. 20

was purchased from Brown Paper Co. All other chemicals used were reagent grade.

Results

Figure 3.1 shows DEAE cellulose chromatograms of the proteolytic activity in three ammonium sulfate fractions from a stationary culture of N. crassa. After DEAE cellulose chromatography, each fraction was assayed at acidic (5.0), neutral (7.0) and alkaline (8.5) pH. Figures 3.1A, 3.1D, and 3.1G show that each ammonium sulfate fraction contains a single alkaline proteolytic activity. Panels B, E, and H of Fig. 3.1 show the activity at neutral pH in the three ammonium sulfate fractions. The 0-40% (Fig. 3.1B) fraction contains multiple activities, whereas the neutral proteolytic activity of the 40-50% (Fig. 3.1E) and the 50-80% (Fig. 3.1H) fractions correspond to the alkaline protease profile. Panels C, F, and I of Fig. 3.1 show the acidic proteolytic activities in the three fractions. The 0-40% (Fig. 3.1C) fraction contains multiple activities (note especially the peak at column fraction 65 and compare with Figs. 3.1A and 3.1B). The 40-50% (Fig. 3.1F) fraction contains two peaks, one of which coincides with the alkaline and neutral peaks of activity. There are multiple peaks in the 50-80% (Fig. 3.1I) fraction.

Figure 3.2 is similar to Fig. 3.1 except that the starting material came from an early log culture of N. crassa. Overall there is reduced activity in the early log vs stationary phase. In addition, the early log 0-40% (Fig. 3.2A) fraction contains no alkaline activity and no acidic activity at column fraction 65 (Fig. 3.2B). The activities in

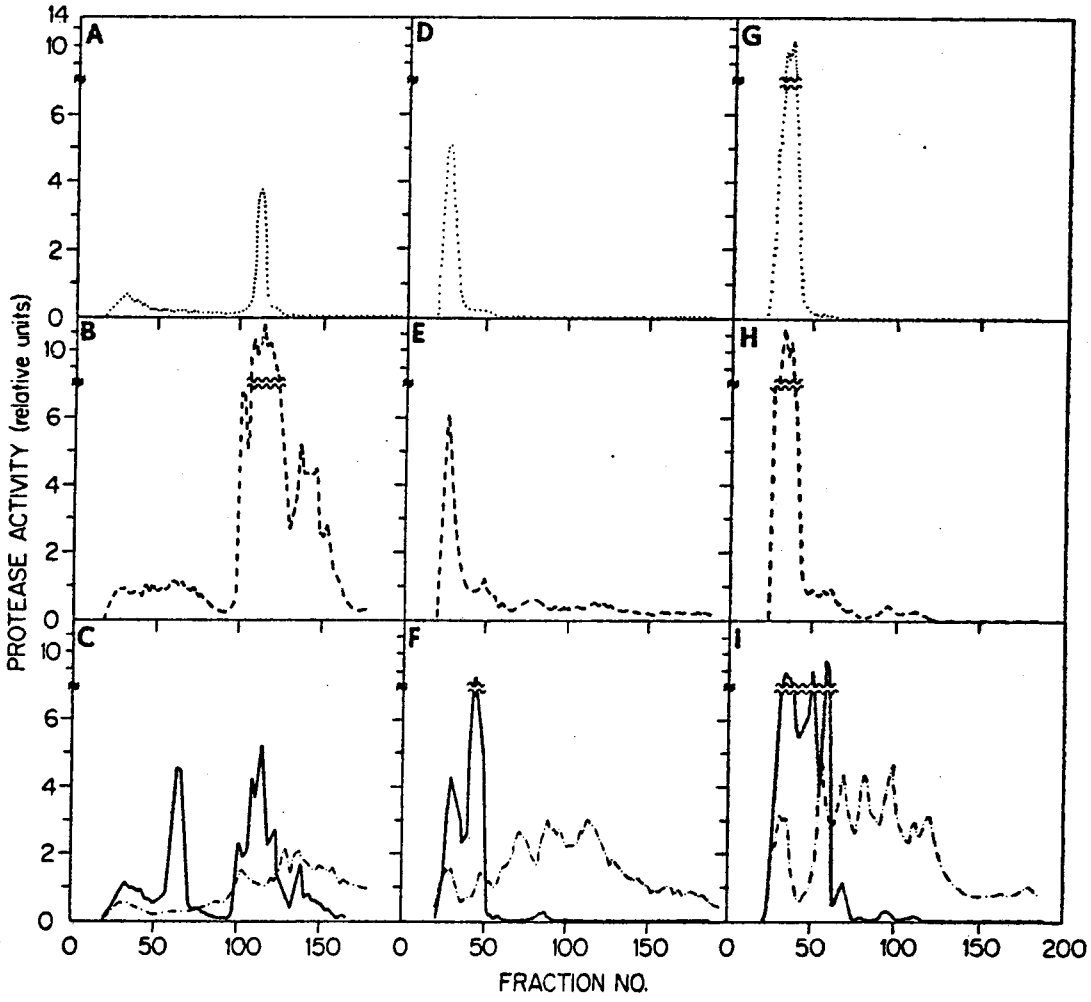

Fig. 3.1. Proteolytic activity in fractions from stationary phase N. crassa. Three ammonium sulfate fractions from a stationary culture were chromatographed on DEAE cellulose as described in Materials and Methods. The chromatographs were assayed for proteolytic activity with azocoll at pH 8.5 (····), pH 7.0 (----), and pH 5.0 (——). The saturation with ammonium sulfate was A, B, C, 0-40%; D, E, F, 40-50%; and G, H, I, 50-80%. Protein (-·-·-) was estimated by absorbance at 280 nm.

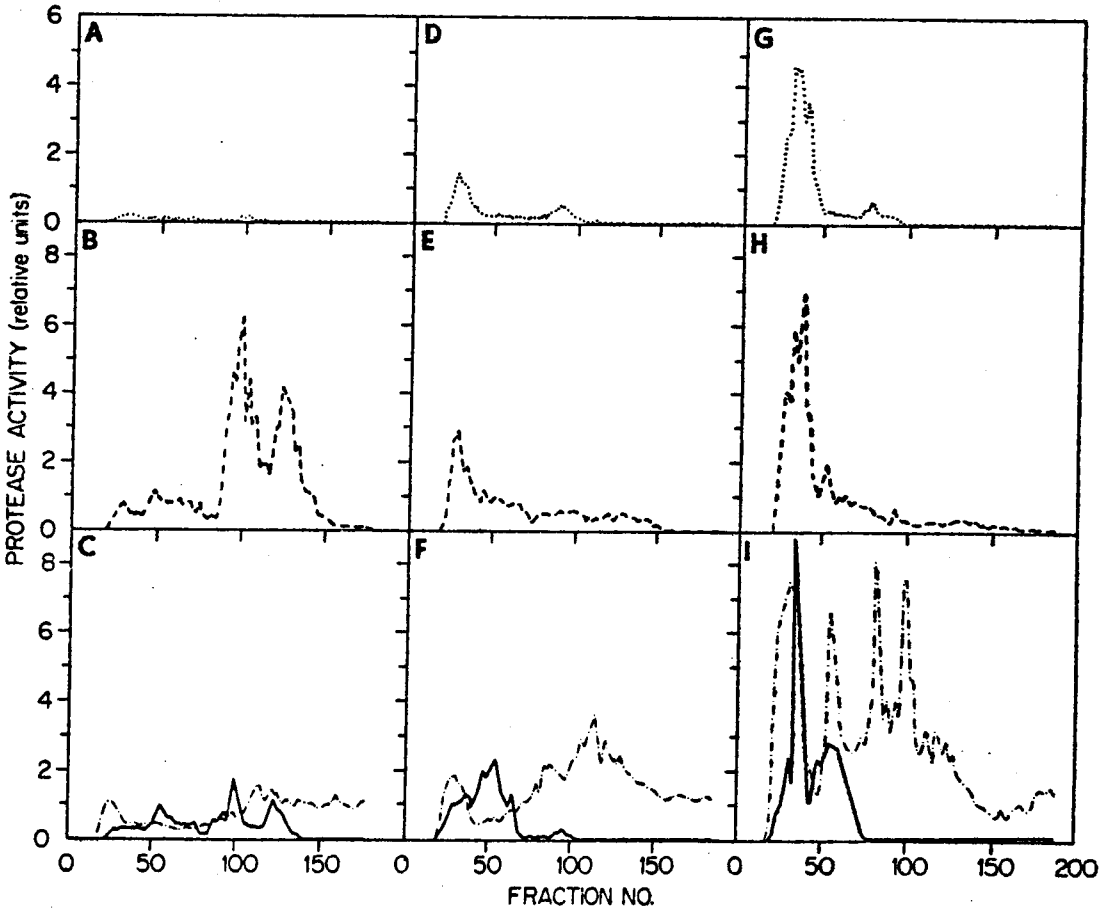

Fig. 3.2. Proteolytic activity in fractions from early log phase N. crassa. See Fig. 3.1 for further description.

the 40-50% (Figs. 3.2D, 3.2E, and 3.2F) ammonium sulfate fractions of the early log culture are moderate to low at each pH assayed. The acidic activity in the 50-80% (Fig. 3.2I) fraction is markedly reduced, while the neutral (Fig. 3.2H) and alkaline (Fig. 3.2G) proteolytic activity profiles of this fraction suggest that it contains multiple enzymes.

The proteolytic activity of stationary phase N. crassa fractions stored at -10°C over a ten month period and at six months after repeated freezing (-20°C) and thawing is compared in Fig. 3.3. Panels A, B, C, and D of Fig. 3.3 show activity in the 0-40% ammonium sulfate fraction. The multiple acidic and neutral proteolytic activities increase significantly from the fresh preparation (Fig. 3.3D) to six months (Fig. 3.3B) and then decrease after ten months (Fig. 3.3A) of storage. The single alkaline peak in the fresh preparation (Fig. 3.3D) increases and broadens during the repeated freezing and thawing (Fig. 3.3C). This trend is even more pronounced after six months storage (Fig. 3.3B). The alkaline activity continues to increase after ten months storage (Fig. 3.3A) when the acidic and neutral proteolytic activities decline. Panels E, F, G, and H of Fig. 3.3 represent the proteolytic activities observed after chromatography of the 40-50% ammonium sulfate fraction. Much of the activity in the fresh preparation (Fig. 3.3H) is lost after freezing and thawing (Fig. 3.3G), but it remains about the same after six and ten months storage (Figs. 3.3F and 3.3E). Column fractions 50-175, which exhibited very slight activity in the fresh preparation (Fig. 3.3H), contained minor proteolytic

Fig. 3.3. The effects of freeze/thaw and storage at -10°C on the proteolytic activity in fractions from stationary phase N. crassa. DEAE cellulose chromatography and ammonium sulfate fractionation were done as shown in Fig. 3.1. The chromatograms were assayed with azocoll at pH 8.5 (····), pH 7.0 (----), and pH 5.0 (——). The saturation with ammonium sulfate was A, B, C, D, 0-40%; E, F, G, H, 40-50%; and I, J, K, L, 50-80%. D, H, and L show proteolytic activity in the three ammonium sulfate fractions immediately after chromatography. D, G, and K show the activity after freezing and thawing a one ml aliquot approximately 10 times over a six month period. B, F, and J show the activity after storage in 20 ml plastic vials for six months at -10°C. A, E, and I show the proteolytic activity after ten months storage under the same conditions.

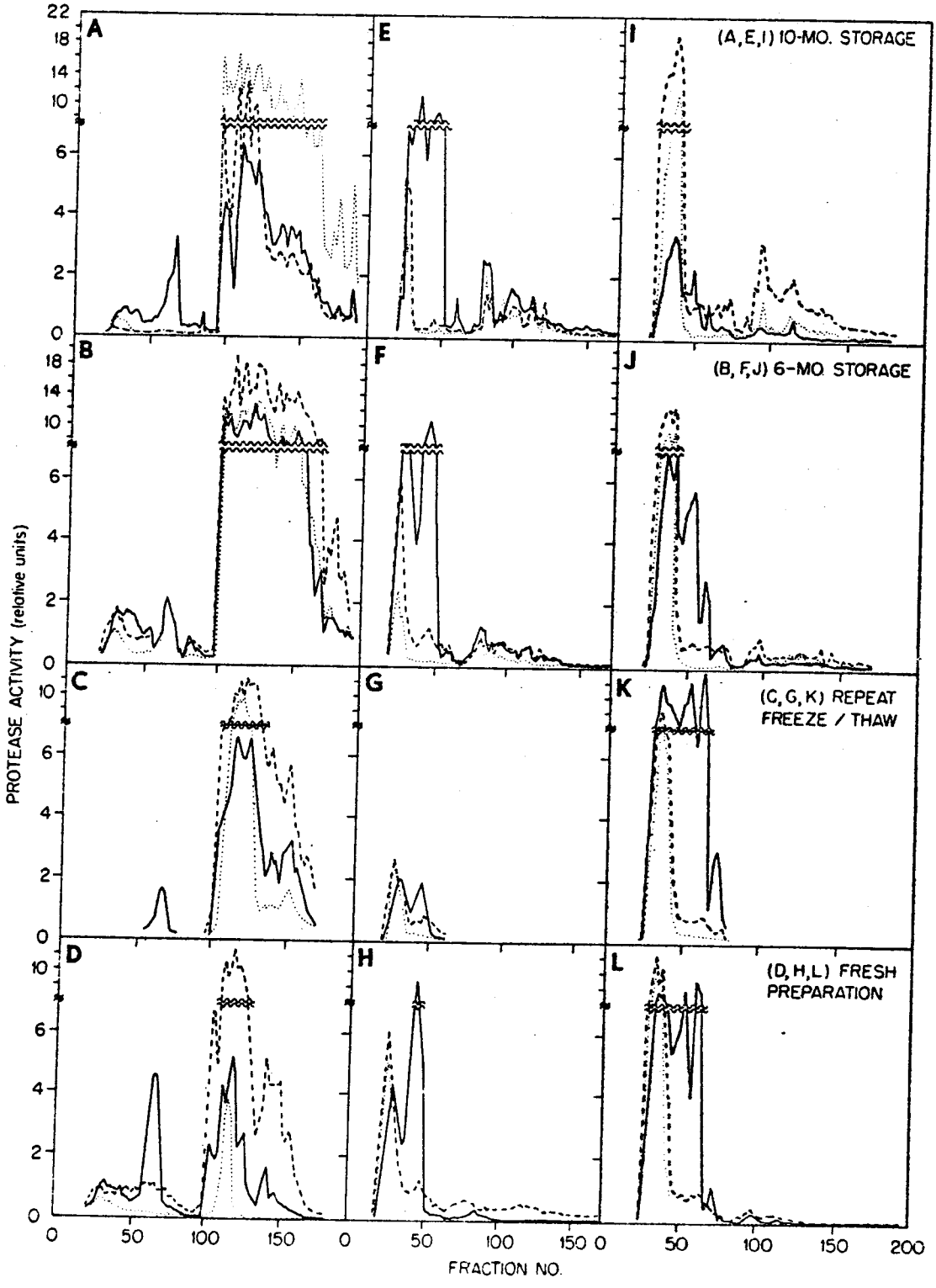
A
E
I
(A,E,I) 10-MO. STORAGE
B
F
J
(B, F,J) 6-MO. STORAGE
C
G
K
(C, G, K) REPEAT FREEZE / THAW
D
H
L
(D, H, L) FRESH PREPARATION
PROTEASE ACTIVITY (relative units)
FRACTION NO.

Fig. 3.3.

activities that increase with time (Figs. 3.3F and 3.3E). Panels I, J, K, and L of Fig. 3.3 show the activity in the 50-80% ammonium sulfate fraction. The fresh preparation profile (Fig. 3.3L) is unchanged by freeze/thaw (Fig. 3.3K) but loses some of the acidic activity after six months storage (Fig. 3.3I). Again, minor proteolytic activities develop during storage.

Figure 3.4 compares proteolytic profiles of early log phase N. crassa fractions over a period of ten months storage at -10°C and at six months after repeated freezing (-20°C) and thawing. Panels A, B, C, and D of Fig. 3.4 show the activity in the 0-40% ammonium sulfate fraction. They display the same characteristics as the corresponding panels in Fig. 3.3 (stationary phase culture), i.e., the acidic and neutral protease activities increase up to six months storage (Fig. 3.4B) and decrease at ten months (Fig. 3.4A), and the alkaline activity steadily increases to become the dominant activity at ten months storage (Fig. 3.4A). However, there are differences between the two growth stages. First, there is no apparent alkaline activity in the fresh preparation of the early log culture (compare Fig. 3.3D with 3.4D). Second, in column fractions 65-80 of the early log phase culture, there are multiple activities at ten months storage (Fig. 3.4A) not seen in the corresponding fresh preparation (Fig. 3.4D). These activities do not appear in the stationary culture in either the fresh preparation or after ten months storage (Figs. 3.3A and 3.3D). Panels E, F, G, and H of Fig 3.4 show the proteolytic activity in the 40-50% ammonium sulfate fraction. Of the six original ammonium sulfate fractions analyzed, the 40-50% fraction of the early log phase culture

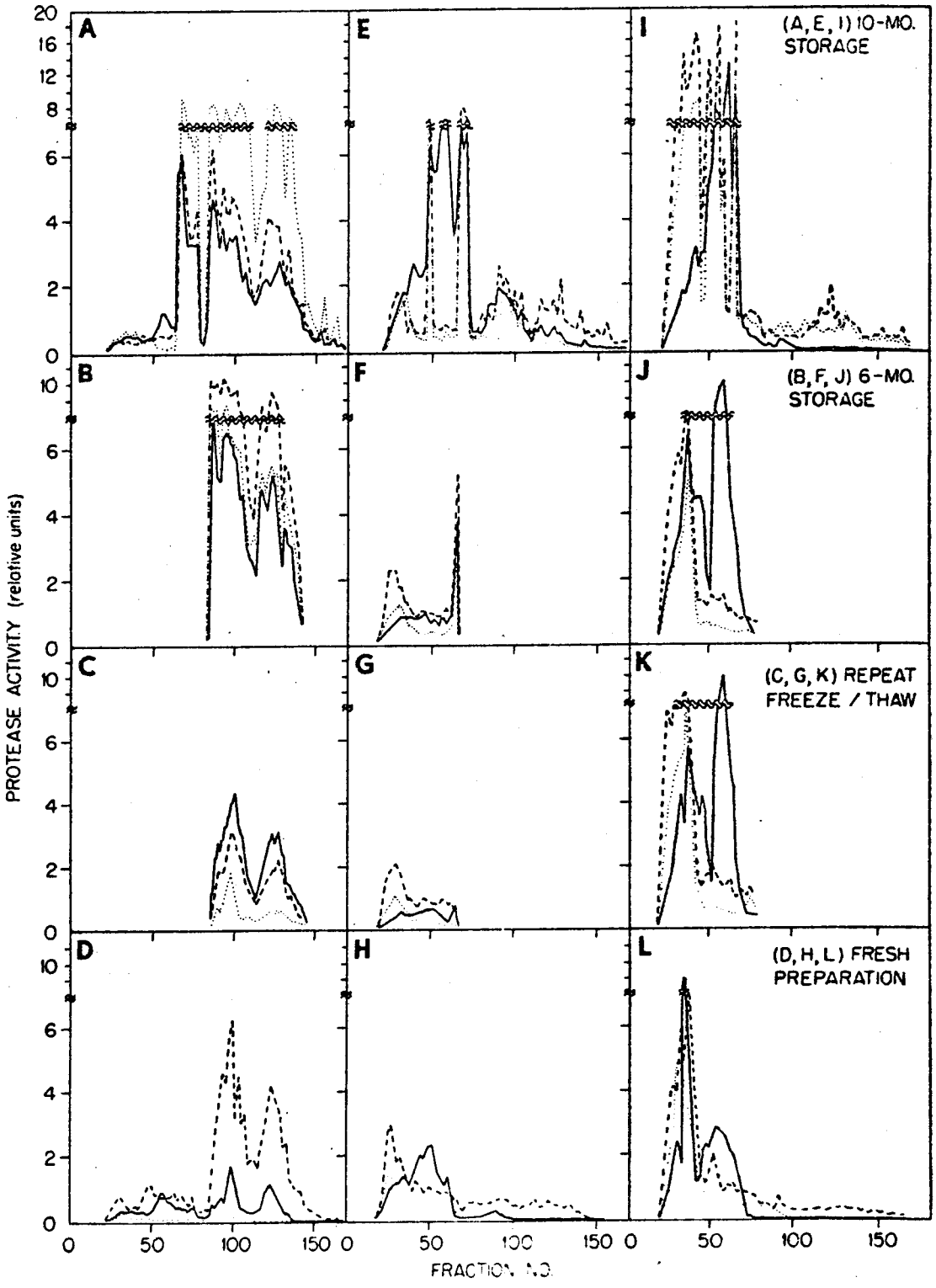

Fig. 3.4. The effects of freeze/thaw and storage at -10°C on the proteolytic activity in fractions from early log phase N. crassa. See Fig. 3.3 for further description.

displays the least activity in the fresh preparation (Fig. 3.4H). Freezing and thawing reduces the small amount of acidic proteolytic activity, whereas the neutral and alkaline proteolytic activities are stable (Fig 3.4G). A single new peak, column fraction 69, appears after six months storage (Fig. 3.4F), and this peak broadens after ten months storage (Fig. 3.4E). In addition, substantial acidic proteolytic activitiy (Fig. 3.4E, column fractions 49-63) and also neutral proteolytic activity (Fig. 3.4E, column fraction 49) are released after ten months storage. There is no comparable release of activities in the stationary phase 40-50% fraction (see Fig. 3.3E). Panels I, J, K, and L of Fig. 3.4 show the activities in the 50-80% ammonium sulfate fraction. In column fractions 20-45, the neutral and alkaline activities increase with time of storage (Figs. 3.4L, 3.4J, 3.4I). In the same region the acidic proteolytic activities are more or less stable at six months (Fig. 3.4J) and then are diminished by ten months storage (Fig. 3.4I). An acidic protease at column fraction 61 is released both by freeze/thaw and by storage (Figs. 3.4K, 3.4J, 3.4I). However the major release of protolytic activities from the 50-80% ammonium sulfate fraction occurs in the regions of chromatographic fractions 47-69. Both the neutral and alkaline proteolytic activities increase substantially after ten months storage (Fig. 3.4I), whereas neither is found to any significant degree at other times in that area of the chromatograms (Figs. 3.4L, 3.4K, and 3.4J). Once again the phenomenon has no counterpart in stationary phase cultures kept under identical conditions (Fig. 3.3I-L). The release of these proteolytic activities in the early log cultures in

all ammonium sulfate fractions examined underscores a difference between early log and stationary phase cultures.

The effects of natural inhibitors on the chromatographic fractions of the 0-40% ammonium sulfate fraction of stationary and early log phase proteolytic activities are shown in Fig. 3.5. Comparison of the effects of Inhibitors 1 through 5 with the controls (Figs. 3.5C, 3.5F, no inhibitor) shows that the profiles change depending upon which class of natural inhibitor is present. Inhibitor 1 (obtained from a 0-40% ammonium sulfate fraction, see Materials and Methods) apparently inhibits all of the proteolytic activities of the 0-40% ammonium fulfate fraction in both early log and stationary cultures (Figs. 3.5B and 3.5E). The remaining panels show that the other natural inhibitors display different effects, i.e., Inhibitor 4 inhibits acidic proteolytic activities in both stationary and early log cultures (Figs. 3.5H and 3.5K), whereas Inhibitor 3 restricts the expression of alkaline activity in the stationary phase to a single peak (Fig. 3.5I). Figures 3.6 and 3.7 show similar experiments with the 40-50% and 50-80% ammonium sulfate fractions, respectively. Again, the different classes of natural inhibitors have varying effects on the profiles. For example, in the 40-50% fraction, Inhibitor 5 has little effect on the stationary acidic activity but does inhibit the alkaline activity (Fig. 3.6G); in the 50-80% ammonium sulfate fraction, Inhibitor 2 inhibits all the early log proteolytic activities but has less effect on the neutral and alkaline activities of the stationary culture (Figs. 3.7A and 3.7D).

Fig. 3.5. The effects of heat-stable natural inhibitors on the proteolytic activity in fractions from 0-40% ammonium sulfate preparations of stationary and early log cultures of N. crassa. DEAE cellulose chromatography and ammonium sulfate fractionation were done as shown in Fig. 3.1. A, B, C, and G, H, I are stationary phase fractions. D, E, F, and J, K, L are early log phase fractions. The chromatograms C and F were assayed with azocoll, minus inhibitor. The remaining chromatograms were assayed in the presence of various natural heat-stable inhibitor preparations: B and E, Inhibitor 1; A and D, Inhibitor 2; I and L, Inhibitor 3; H and K, Inhibitor 4; and G and J, Inhibitor 5. Natural heat-stable inhibitors were obtained from 0-40% (Inhibitor 1), 40-50% (Inhibitor 2), 50-60% (Inhibitor 3), 60-70% (Inhibitor 4), and 70-80% (Inhibitor 5) ammonium sulfate fractions prepared as described in Materials and Methods. Assays were done at pH 8.5 (....), pH 7.0 (----), and pH 5.0 (——).

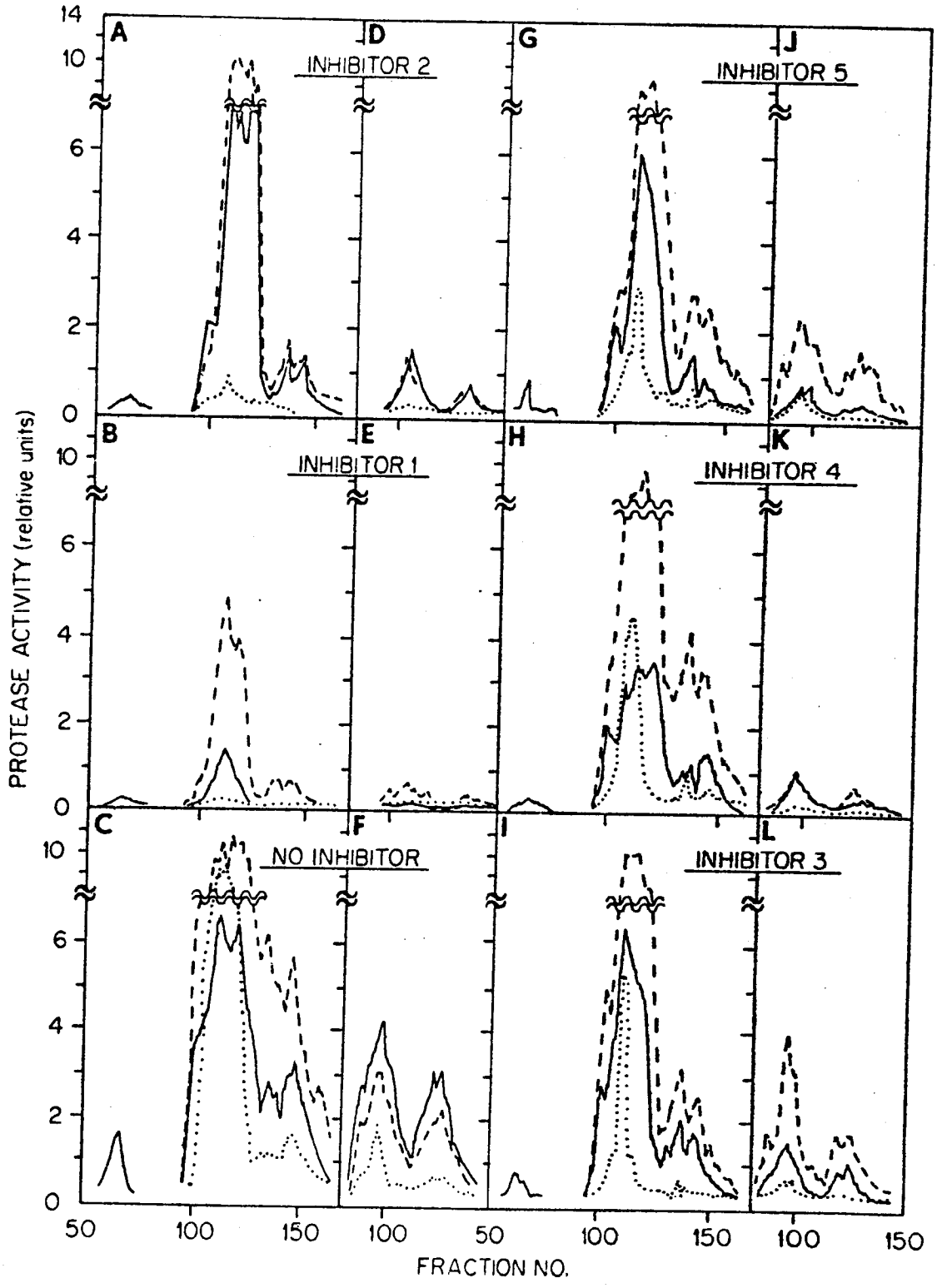
A
D
INHIBITOR 2
G
J
INHIBITOR 5
B
E
INHIBITOR 1
H
K
INHIBITOR 4
C
F
NO INHIBITOR
I
L
INHIBITOR 3
PROTEASE ACTIVITY (relative units)
FRACTION NO.
0
2
4
6
10
14
50
100
150

Fig. 3.5.

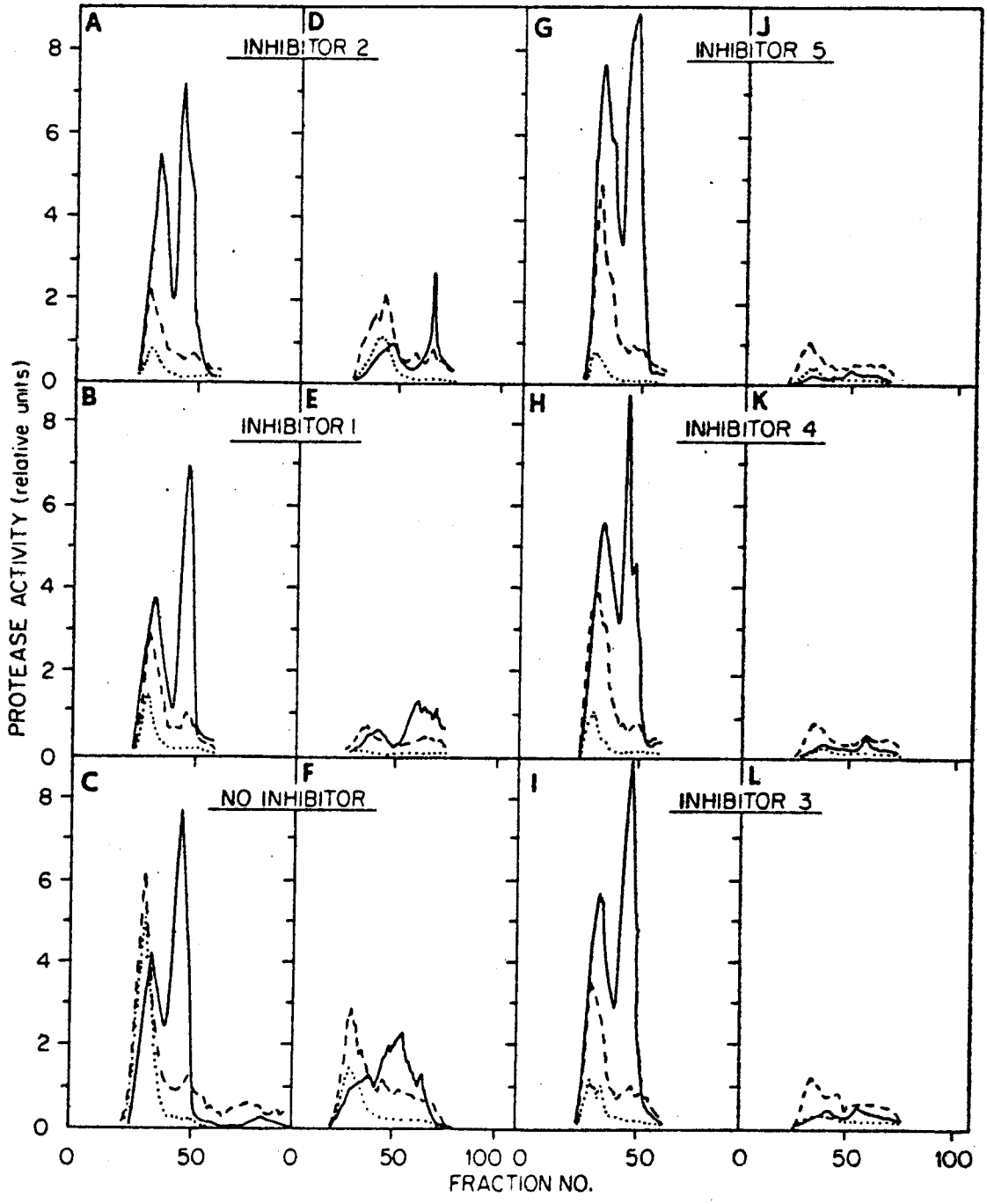

Fig. 3.6. The effects of heat-stable natural inhibitors on the proteolytic activity in fractions from 40-50% ammonium sulfate preparations of stationary and early log cultures of N. crassa. See Fig. 3.5 for further description.

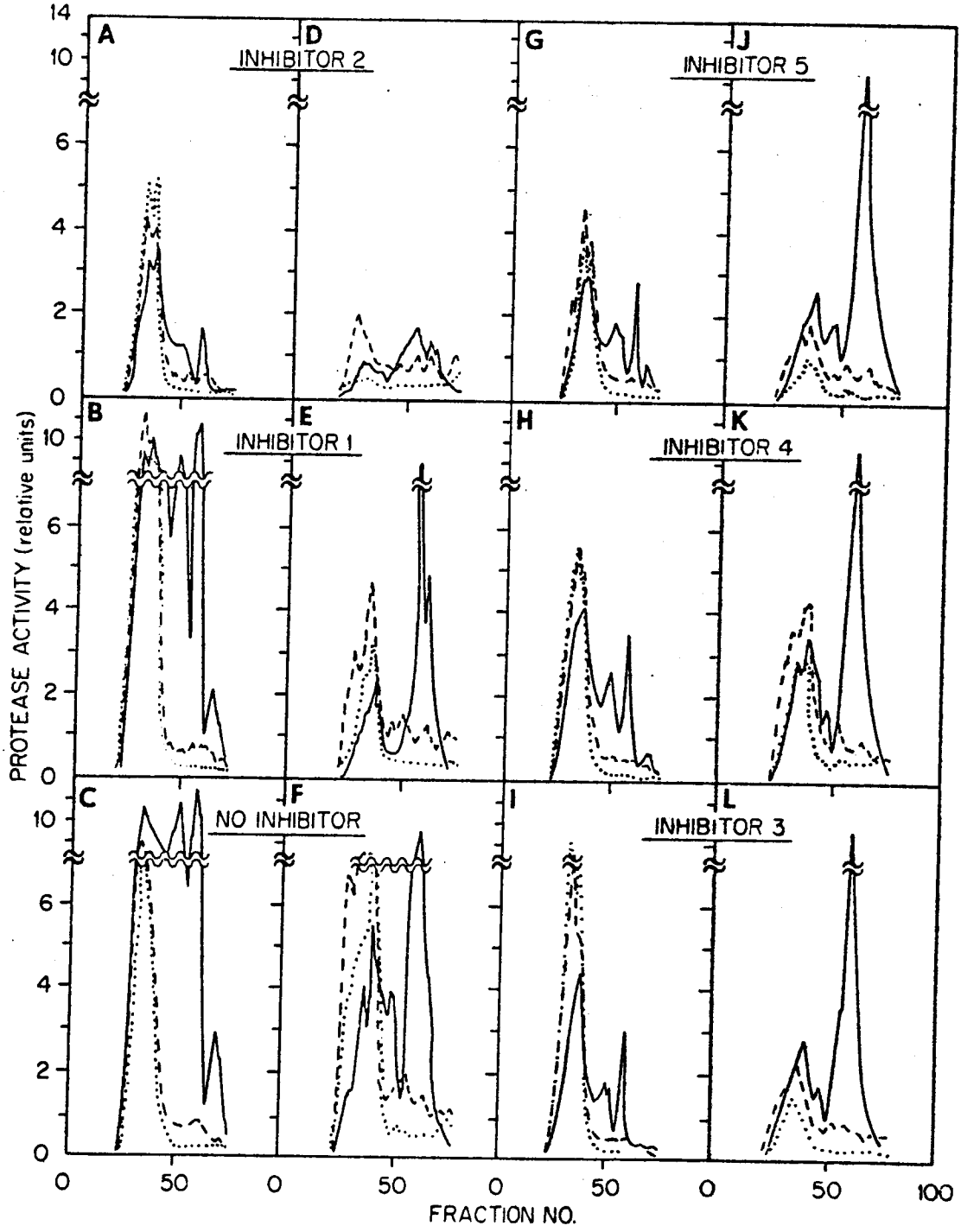

Fig. 3.7. The effects of heat-stable natural inhibitors on the proteolytic activity in fractions from 50-80% ammonium sulfate preparations of stationary and early log cultures of N. crassa. See Fig. 3.5 for further description.

Figure 3.8 shows the effects of commercial inhibitors on the proteolytic activity in the 0-40% ammonium sulfate fraction of stationary and early log phase cultures. PCMB (Figs. 3.8B and 3.8E) is evidently a good inhibitor of all the activities. PMSF (Figs. 3.8A and 3.8D) also inhibits all of the activities, although some stationary phase neutral and alkaline proteolytic activities withstand the effects of PMSF at this concentration. Benzamidine causes a curious enhancement of the acidic proteolytic activity of column fraction 65 (Fig. 3.8I) in the stationary phase culture. Benzamidine also inhibits acidic activities in both stationary and early log fractions and reduces all alkaline and neutral activity in the stationary culture except column fraction 115 (Figs. 3.8I and 3.8L). A single peak of acidic proteolytic activity appears to be unaffected by Trasylol (Figs. 3.8H and 3.8K). The effects of Inhibitor 1 (from Figs. 3.5B and 3.5E) are included for comparison.

The effects of commercial inhibitors on the proteolytic activity profile of the 40-50% ammonium sulfate fractions of stationary and early log phase cultures are shown in Fig. 3.9. In the stationary fraction PCMB (Fig. 3.9B) inhibits the alkaline activity and one of the acidic activities (column fraction 45). The other acidic activity and the neutral activity appear to be resistant to the effects of PCMB. PMSF (Figs. 3.9A and 3.9D) does not affect the alkaline activity of either stationary or early log cultures. Benzamidine moderately inhibits all activities in the stationary culture but has little effect on those of the early log culture (Fig. 3.9I and 3.9L). Trasylol primarily inhibits the alkaline stationary phase activity (Fig. 3.9H)

Fig. 3.8. The effects of commercial inhibitors on the proteolytic activity in fractions from 0-40% ammonium sulfate preparations of stationary and early log cultures of N. crassa. DEAE cellulose chromatography and ammonium sulfate fractionation were done as shown in Fig. 3.1. A, B, C, and G, H, I are stationary phase fractions. D, E, F, and J, K, L are early log phase fractions. The chromatograms C and F were assayed with azocoll, minus inhibitor. The remaining chromatograms were assayed in the presence of various commercial inhibitors: B and E, PCMB (1 mM); A and D, PMSF (1 mM); I and L, benzamidine (1 mM); and H and K, Trasylol (100 units). In chromatograms G and J the effects of Inhibitor 1 from Fig. 3.5 are included for comparison. Assays were done at pH 8.5 (····), pH 7.0 (----), and pH 5.0 (——).

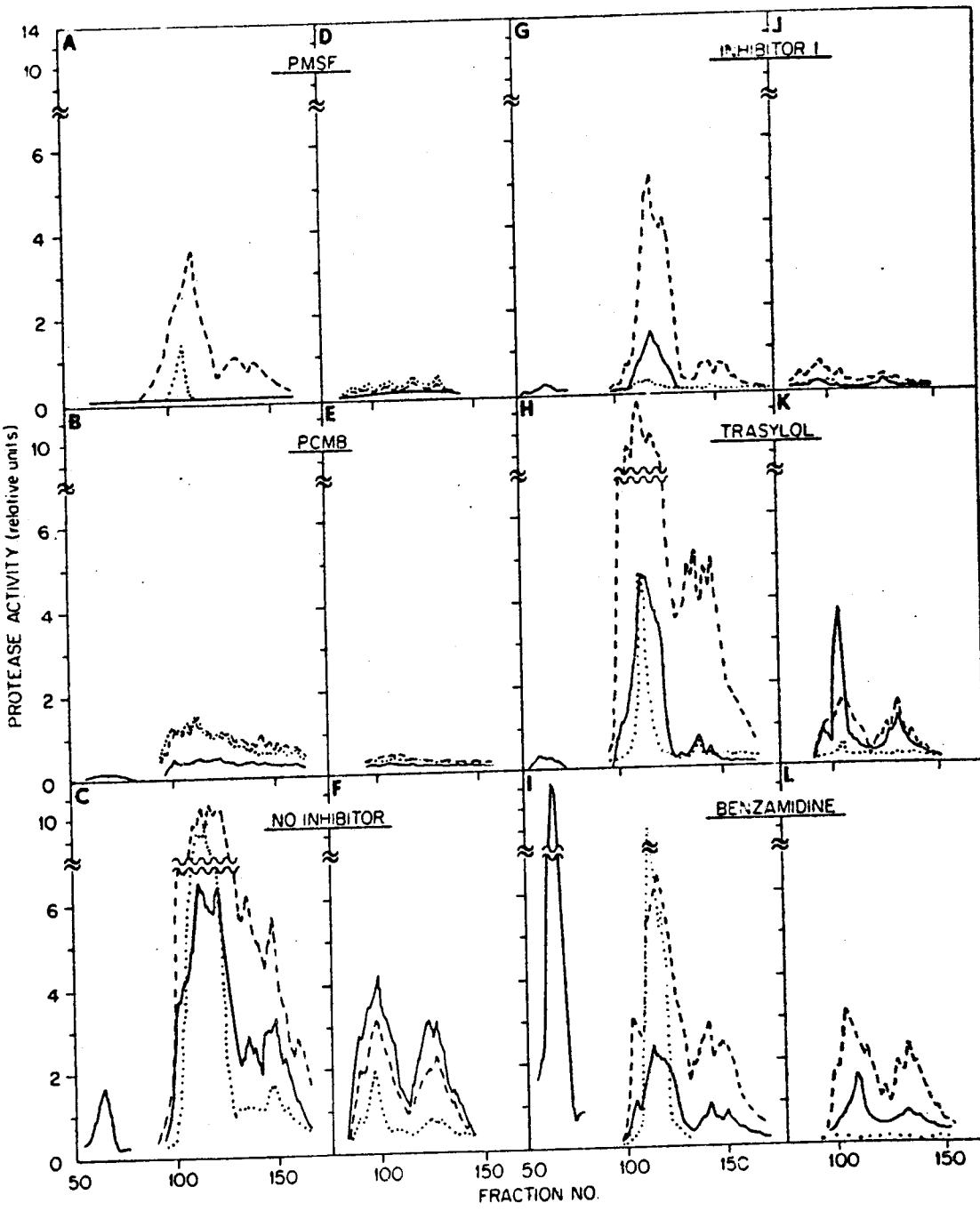
A
D
PMSF
G
J
INHIBITOR I
B
E
PCMB
H
K
TRASYLOL
C
F
NO INHIBITOR
I
L
BENZAMIDINE
PROTEASE ACTIVITY (relative units)
FRACTION NO.
0
2
4
6
10
14
50
100
150

Fig. 3.8.

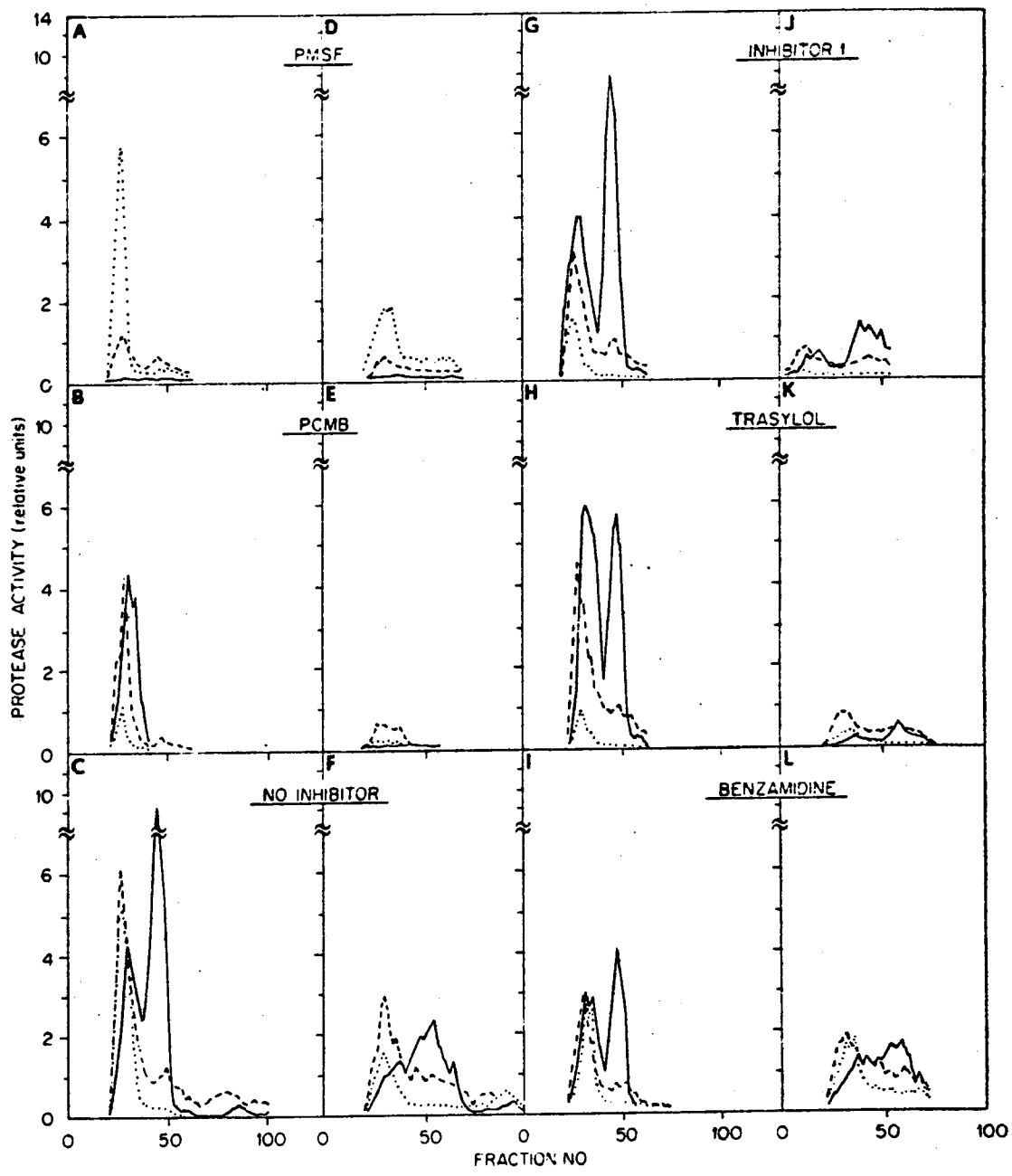

Fig. 3.9. The effects of commercial inhibitors on the proteolytic activity in fractions from 40-50% ammonium sulfate preparations of stationary and early log cultures of N. crassa. Description is the same as in Fig. 3.8 except that chromatograms G and J show the effects of Inhibitor 1 from Fig. 3.6 for comparison.

and all early log activities (Fig. 3.9K). The effects of Inhibitor 1 (from Figs. 3.6B and 3.6E) are again included from comparison.

Figure 3.10 shows the effects of commercial inhibitors on the proteolytic activity of the 50-80% ammonium sulfate fractions of stationary and early log phase cultures. PCMB (Figs. 3.10B and 3.10E) inhibits all activities in both stationary and early log phase fractions. PMSF (Fig. 3.10A and 3.10D) inhibits all acidic activities in both cultures but is ineffective against the alkaline activity. Neutral activity is inhibited more in the early phase by PMSF than in the stationary phase. Benzamidine has no effect on the activity in the stationary phase culture but does inhibit both neutral and alkaline activity in the early log phase culture (Figs. 3.10I and 3.10L). In the stationary culture Trasylol (Fig. 3.10H) inhibits all activities, although column fractions 20-45 are resistant at each pH. In the early log culture Trasylol has little effect on the neutral activity and only a moderate effect on the alkaline activity (Fig. 3.10K). The acidic activity is also moderately inhibited except in column fraction 61, where no inhibition is seen. The effects of Inhibitor 2 (from Figs. 3.7A and 3.7D) are included for comparison.

Table 3.1 gives a conservative tabulation of the proteases of N. crassa. This compilation was made from careful examination and comparison of the proteolytic activity profiles shown in the figures. Each protease listed has properties that distinguish it from others in Table 3.1. There are a great number of shoulders on different proteolytic peaks that suggest still more distinct proteases not separated by the chromatography, but these are not included in the

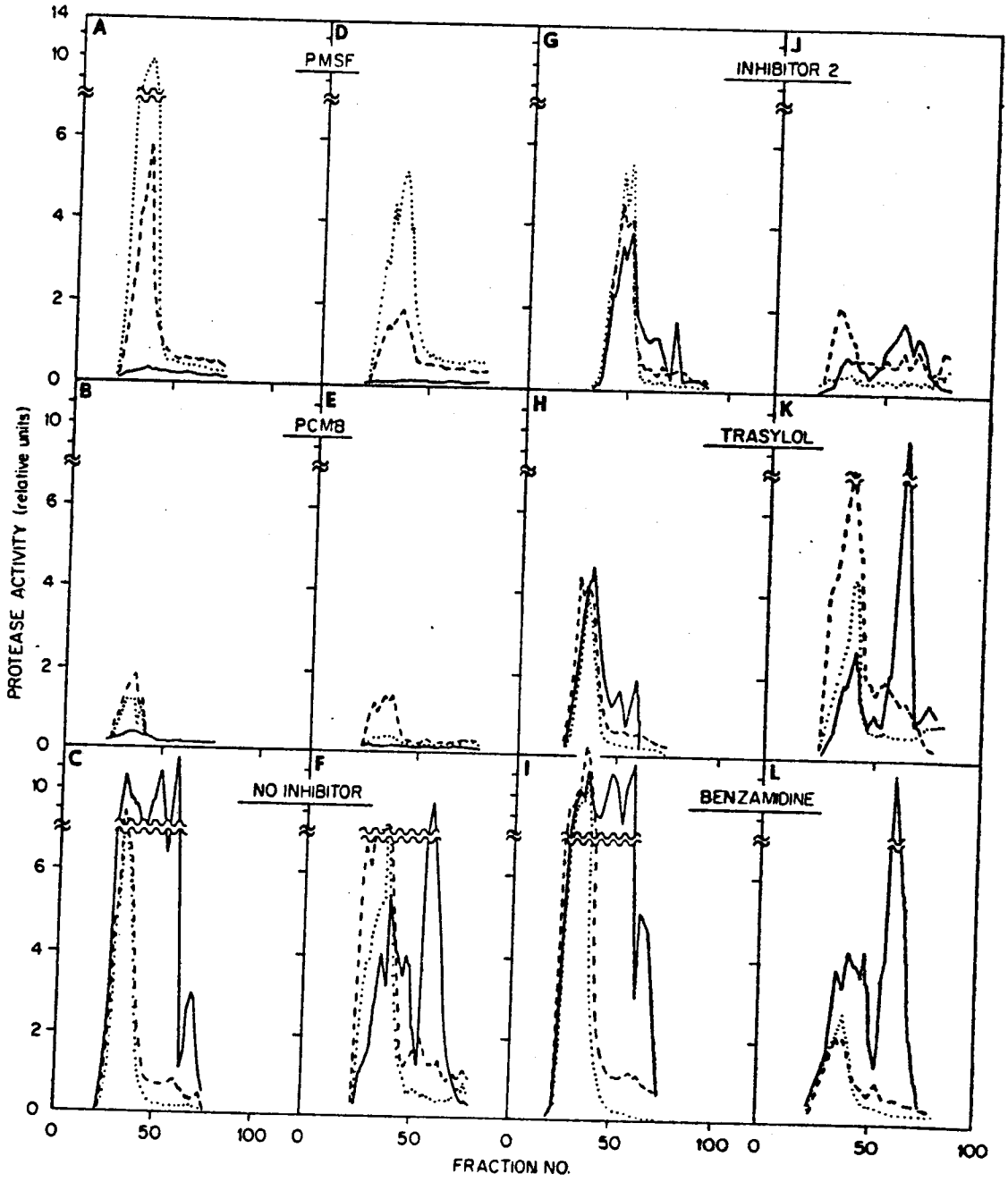

Fig. 3.10. The effects of commercial inhibitors on the proteolytic activity in fractions from 50-80% ammonium sulfate preparations of stationary and early log cultures of N. crassa. Description is the same as in Fig. 3.8 except that chromatograms G and J show the effects of Inhibitor 2 from Fig. 3.7 for comparison.

TABLE 3.1. Distinguishing Properties of the Multiple Proteases in N. crassa

Protease[a]	Figure	$(NH_4)_2SO_4$ Fraction	DEAE cellulose Fraction	pH of Assay	Distinguishing Properties[b]
1	3.3D	0-40%	63	5.0	Chromatographic location (3.3E), activation by benzamidine (3.8I)
2	3.3D	0-40%	113	8.5	Inhibited by Inhibitor 2 (3.5A), activated by 6-month storage (3.3B)
3	3.3D	0-40%	101	7.0	Inhibited by Inhibitor 2 (3.5A), Inhibited by Inhibitor 5 (3.5G)
4	3.3D	0-40%	101	5.0	Inhibited by trasylol (3.8H)
5	3.3D	0-40%	105-125	5.0	Inhibited by Inhibitor 4 (3.5H), less resistant to trasylol than pH 7.0 activity in area (3.8H)
6	3.3D	0-40%	105-125	7.0	Trasylol resistant (3.8H), Inhibitor 4 resistant (3.5H)
7	3.3D	0-40%	127-149	7.0	Trasylol resistant (3.8H)
8	3.3D	0-40%	127-149	5.0	Inhibited by trasylol (3.8H)
9	3.3H	40-50%	27	8.5	PMSF resistant (3.9A), decays with storage (3.3E, F, G, H)

TABLE 3.1 (continued)

Protease[a]	Figure	$(NH_4)_2SO_4$ Fraction	DEAE cellulose Fraction	pH of Assay	Distinguishing Properties[b]
10	3.3H	40-50%	27	7.0	PCMB resistant (3.9B), stable with storage (3.3E, F, G, H), inhibited by Inhibitor 2 (3.6A)
11	3.3H	40-50%	31	5.0	PCMB resistant (3.9B)
12	3.3H	40-50%	45	5.0	Inhibited by benzamidine (3.9I), and PCMB (3.9B), decays during freeze/thaw (3.3G)
13	3.3L	50-80%	24-40	7.0, 8.5	PMSF resistant (3.10A), activated by benzamidine (3.10I)
14	3.3L	50-80%	24-43	5.0	Decays with storage (3.3I), Inhibited by PMSF (3.10A)
15	3.3L	50-80%	43-63	5.0	Inhibited by trasylol (3.10H)
16	3.3L	50-80%	69	5.0	Activated by benzamidine (3.10I)
17	3.3D	0-40%	150	8.5	No initial activity (3.3D), released during freeze/thaw (3.3C), multiple peaks after storage (3.3B, 3.3A)
18	3.4A	0-40%	63-79	5.0, 7.0, 8.5	Appearance after 10 month storage (3.4A), no comparable stationary phase activity (3.3A)

TABLE 3.1 (continued)

Protease[a]	Figure	$(NH_4)_2SO_4$ Fraction	DEAE cellulose Fraction	pH of Assay	Distinguishing Properties[b]
19	3.4H	40-50%	23-39	7.0	Inhibitor 2 resistant (3.6D)
20	3.4E	40-50%	49	5.0, 7.0, 8.5	Same as No. 18
21	3.4E	40-50%	65-73	5.0, 7.0, 8.5	Same as No. 18
22	3.4L	50-80%	25-45	7.0, 8.5	Trasylol resistant (3.10K), inhibited by benzamidine (3.10L)
23	3.4K	50-80%	61	5.0	Activated by freeze/thaw (3.4K), stable with storage (3.4I, 3.4J), Trasylol resistant (3.10K)
24	3.4I	50-80%	47-51	7.0, 8.5	Same as No. 18
25	3.4I	50-80%	51-55	5.0, 7.0, 8.5	Same as No. 18
26	3.4I	50-80%	63-69	5.0, 7.0, 8.5	Same as No. 18

[a]Growth phase for 1-17 is stationary; for 18-26, early log.

[b]Figure numbers in parentheses

tabulation nor have we included the minor proteolytic activities expressed after ten months storage.

Discussion

In our survey of proteases from N. crassa, we looked for differences in the proteolytic activities of early log and stationary phase cultures. Such differences do exist (compare Figs. 3.3, pg 24, and 3.4, pg 27), but their importance was eclipsed as the study progressed by the unexpected number and abundance of proteases in both preparations, and by the continual expression of new and increased proteolytic activities after long-term storage. The summary in Table 3.1 shows that 26 proteases in N. crassa were distinguishable from one another.

Many questions arise from examination of the detailed data presented in the figures. For example, why is there such a profound increase in alkaline activity, especially in the 0-40% ammonium sulfate fraction, after storage for six months? Does this represent loss of natural inhibitors? If so, why don't the inhibitors show their effects at a lower pH? Are there sets of inhibitors active at different pH's? We are also puzzled by coincident activities within a given chromatographic area at different pH's. This occurs frequently and one is tempted to interpret it as an indication that the same protease is active at all the pH's tested. However, close inspection shows that in some instances particular inhibitors affect the proteolytic activity differently, depending on pH. The differences cannot be caused by inactivation of an inhibitor at a particular pH because the same inhibitor can clearly affect other proteolytic

activities at the same pH (see Table 3.1, protease numbers 3-10). The data support the conclusion that the activities seen at different pH often represent different proteases that happen to coincide at a particular chromatographic position.

Still another question arises in the interpretation of the new activities expressed after storage, particularly in the DEAE cellulose fractions from the early logarithmic growth stage cultures (see Figs. 3.3, pg 24, and 3.4, pg 27, panels A, E, and I). Does this expression represent proteolytic activity uncovered as a result of preferential inactivation of inhibitors or possibly the activation of zymogens? Baumgartner and Chrispeels (7) found disappearance of an inhibitor and activation of an endopeptidase in the cotyledons of mung beans when crude fractions were allowed to incubate at 6°C. However, the inhibitory activity was lost much more rapidly than the proteolytic activity increased, which led them to conclude that the events were not causally related.

One may also ask why there is a difference between the activity of the original one ml aliquots repeatedly frozen and thawed for six months and the activity expressed after six months of storage. The fractions stored for six months at -10°C contained 11 ml of solution in plastic vials, whereas the aliquots repeatedly frozen and thawed at -20°C contained one ml or less in glass test tubes. It is possible that the combination of large volume and plastic enclosure provided a slow cooling rate at -10°C, thus offering relatively favorable conditions for protease activation.

In some of the chromatograms presented here there are no fractions entirely devoid of proteolytic activity (see Figs. 3.3, pg 24, and 3.4, pg 27). This underlines the importance of being aware of potential proteolysis during routine protein biochemistry and presents a significant challenge to the investigator. It also offers a simple interpretation of why so many "purified" proteins are unstable. Pringle (38) forcefully pointed this out in his examination of 40 highly purified, unstable, yeast enzymes; all were found to contain proteolytic contaminants. Proteolytic attack need not inactivate an enzyme but can, instead, alter enzymic structure and function *in vitro*. Therefore, if all organisms contain an array of proteases, modifications of proteins during isolation procedures could easily lead to artifacts.

Finally, why does *N. crassa* have so many proteases? There seems to be no *a priori* reason why *N. crassa* should contain inordinately more proteases than any other organisms; this leads to the conclusion that other species may contain similar numbers. If this is true, then it is likely that organisms in general contain a large variety of specific proteases available for various metabolic and regulatory functions. These functions might also be involved in the process of differentiation to a much higher degree than previously conceived or in other, as yet unknown, regulatory roles.

CHAPTER IV

THE CRYPTIC BEHAVIOR OF *NEUROSPORA* PROTEASES

Summary

An early logarithmic growth phase culture of *Neurospora crassa* was fractionated with ammonium sulfate, chromatographed on diethylaminoethyl cellulose, and assayed for intracellular proteolytic activities after storage at various conditions. Storage at 4°C over a period of 23 days gradually releases proteolytic activity throughout the column fractions, with the notable exception of one region that was shown to be enriched with natural protease inhibitors. A series of rapid freezes (-196°C) and thaws releases little proteolytic activity, whereas storage at -15°C provides conditions suitable for the slow release of proteolytic activity. We conclude that most, if not all, of these intracellular proteolytic activities are cryptic in nature.

Introduction

Proteases pose intriguing problems for biochemists (5, 55). Not only are their functions within cells complex, but their *in vitro* behavior is a source of unwanted, and often unheeded, artifacts (38). Frequently, this action is prevented to some degree by the presence of natural inhibitors (23), or it may be partially controlled by the addition of commercial inhibitors (51). In an earlier report (20) we noted that *Neurospora crassa* apparently contains many more proteases than have been previously recognized for this organism or for others, and that many of these proteases became assayable only after

prolonged storage. One of the parameters we have chosen to investigate further is the influence of storage conditions on the release of N. crassa proteolytic activities. Here we report that rapid freezing at -196°C essentially stabilizes the initial activity, whereas storage at 4°C releases substantial amounts of cryptic proteolytic activity.

Materials and Methods

Growth of organism

N. crassa was grown as described earlier (19) and harvested at an early logarithmic stage of growth (7 g/l wet weight). The mycelia were lyophilized, ground in a Wiley mill, and stored at -20°C.

Protein fractionation

Lyophilized ground mycelia (150g) were mixed in 0.01 M potassium phosphate, pH 7.0, for 30 min and centrifuged at 10000 x g for 30 min. Nucleic acids were precipitated by stirring the supernatant with protamine sulfate (0.21% vol) for 15 min and then centrifuging it at 10000 x g for 15 min. Fine crystals of ammonium sulfate were slowly added to the supernatant to 40% saturation (0-40% ammonium sulfate fraction), dissolved, mixed for 15 min, and centrifuged at 10000 x g for 15 min. The ammonium sulfate pellet was dissolved in a minimal volume of phosphate buffer. All preparations were done at 4°C.

Chromatography

The dissolved ammonium sulfate fraction was placed on a 4 X 80 cm column of Sephadex G-25 equilibrated with 0.01 M potassium phosphate, pH 7.0, and the protein-containing eluent (volume ~300 ml) was pumped

onto a 2 X 80 cm column of DEAE celluose equilibrated with 0.01 M potassium phosphate, pH 7.0. The column was eluted with a 0.01-0.25 M gradient of NaCl in 0.01 M potassium phosphate, pH 7.0. Fractions (12 ml each) were collected until the gradient was complete.

Storage of column fractions

From each of the odd-numbered fractions, two sets of one ml aliquots were taken. One set was left to incubate at 4°C. The other set was frozen in liquied nitrogen (-196°C) and stored at -20°C. The remaining volume of the column fractions was stored at -15°C in 20 ml plastic vials.

Azocoll assay

Assays for proteolytic activity, using azocoll as the substrate, were carried out as previously reported (47).

Inhibitor assay

Undiluted inhibitor (0.03 ml) was added to 0.47 ml azocoll reaction mixture (the inhibitor source was fractions 73-87 from the DEAE cellulose column), and 0.02 ml of the protease (fractions 59, 95, and 109 from the DEAE cellulose column) was added to initiate the reaction. The remainder of the assay was done as described above.

Chemicals

Azocoll was purchased from Calbiochem. DEAE cellulose Type 20 was purchased from Brown Paper Co. All other chemicals used were reagent grade.

Results

Incubation at 4°C over a period of 23 days releases increasing levels of proteolytic activity from the DEAE cellulose column fractions. Figure 4.1 shows the proteolytic activity, measured at three pH's, after different times of incubation at 4°C. Comparison of each panel with the initial activity (Fig. 4.1D) reveals a gradual increase in proteolytic activity over the entire time course. Column fractions 73-87 show a marked absence of proteolytic activity. The possibility that this region of the column contained endogenous inhibitor was examined by assay of these fractions for their inhibitory capacity (Fig. 4.2). Of interest is the fact that fractions from this area of the column completely inhibit three distinct peaks of proteolytic activity (fractions 59, 95, and 109).

In Fig. 4.1 the activities may be roughly divided into early and late fractions by the location of the region dominated by inhibitor. Activity in the later fractions (89-189) predominates through the first five days of incubation; only one major proteolytic peak is evident in the early fractions (1-73) of the column at this time. After nine days or more of incubation, other peaks of activity appear in these early fractions, whereas the activity in the later fractions remains relatively constant.

The possibility that some of the effects observed could be the result of microbial activity was considered; microscopic examination of the samples after 23 days of storage showed no contamination of fractions. Also, the addition of 0.02% azide to the samples prior to incubation at 4°C did not alter the results (data not shown).

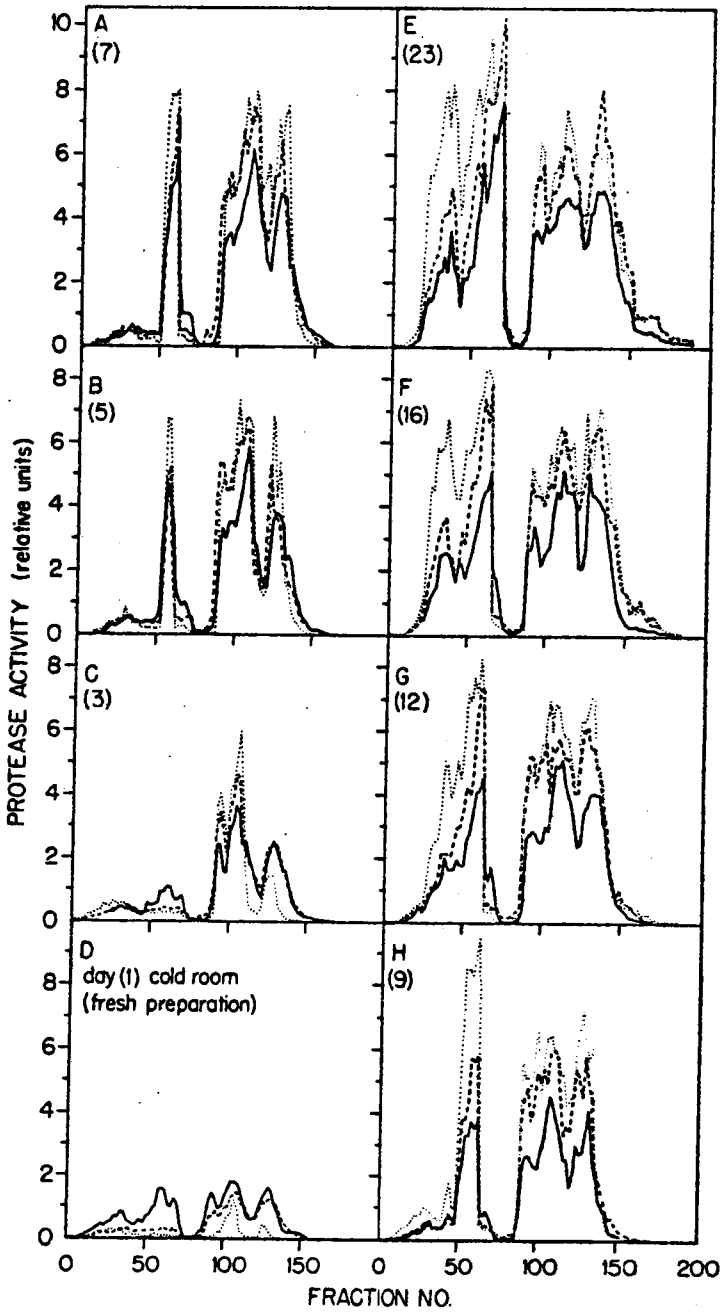

Fig. 4.1. Proteolytic activities from an early logarithmic growth stage of N. crassa and their release during storage at 4°C. Ammonium sulfate fractionation and DEAE cellulose chromatography were done as described in Materials and Methods. The chromatograms were assayed for protolytic activity with azocoll at pH 8.5 (····), pH 7.0 (----), and pH 5.0 (——). Panel D shows the proteolytic activity assayable immediately after chromatography. The remaining panels show the activity present after incubation at 4°C for the number of days indicated (in parentheses).

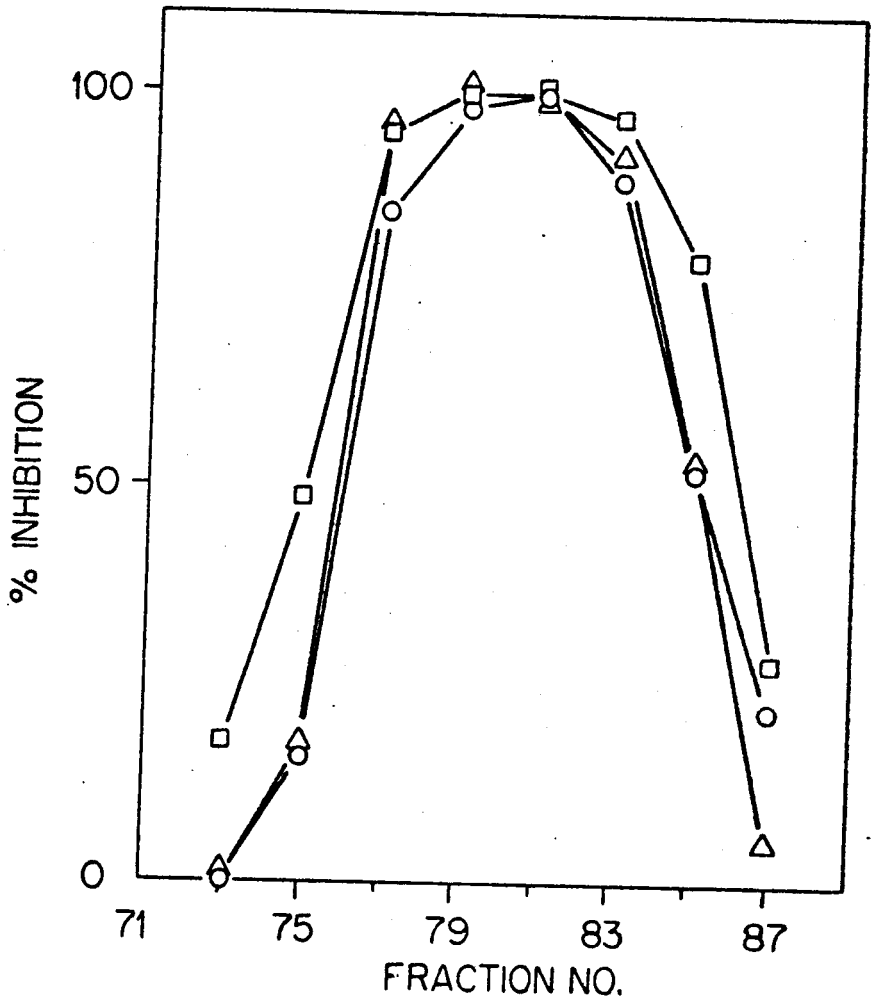

Fig. 4.2. Inhibition of proteolytic activities from an early logarithmic growth phase of N. crassa by incubation in the presence of fractions 73-87 from the DEAE cellulose column. The inhibition assay was done as described in Materials and Methods. The source of protease was fraction 59 (○), fraction 95 (□), and fraction 109 (△) the DEAE cellulose column.

The proteolytic activity of the fractions which were rapidly frozen and thawed, then subsequently allowed to incubate at 4°C for several days, is shown in Fig. 4.3. The level of proteolytic activity remains relatively constant through ten rapid freezes at -196°C and thaws at 25°C. After the fourth rapid freeze/thaw, half of each aliquot was repeatedly frozen and thawed a total of ten times and then assayed for activity. The other half was allowed to incubate at 4°C for several days and assayed periodically. The resulting profiles compare favorably with those of fractions which were incubated at 4°C without being first frozen and thawed (compare Figs. 4.1C and 4.1G with 4.3G and 4.3F).

After the original two sets of one ml aliquots were removed and placed under their particular storage conditions, the remaining volume of each fraction was stored in plastic 20 ml vials, which were then placed in a -15°C freezer. These fractions were periodically thawed and assayed for proteolytic activity at three pH's; the results are shown in Fig. 4.4. A major difference between activities found under these conditions and those found after incubation at 4°C is the rapid appearance of the activity in fractions 55-61.

Discussion

Previously we reported that *N. crassa* appeared to contain a multiplicity of proteases (20) distinguishable from one another on the basis of chromatographic location, pH optima, location within a particular ammonium sulfate preparation, growth stage of the organism, sensitivity to the action of commercial and natural inhibitors,

Fig. 4.3. Stabilization of proteolytic activity from an early logarithmic growth phase of N. crassa during rapid freeze (-196°C)/ thaw (25°C) and subsequent release of activity during incubation at 4°C. Ammonium sulfate fractionation and DEAE cellulose chromatography were done as shown in Fig. 4.1. The chromatograms were assayed for proteolytic activity with azocoll at pH 8.5 (····), pH 7.0 (----), and pH 5.0 (——). Panel E shows the proteolytic activity assayable immediately after chromatography. Panels D, C, B, A, and H show activities for material rapidly frozen (-196°C) and thawed (25°C) one, two, three, four, and ten times, respectively. Half the material which had been frozen and thawed four times was incubated at 4°C for two days (Panel G) and ten days (Panel F) prior to assay for proteolytic activity.

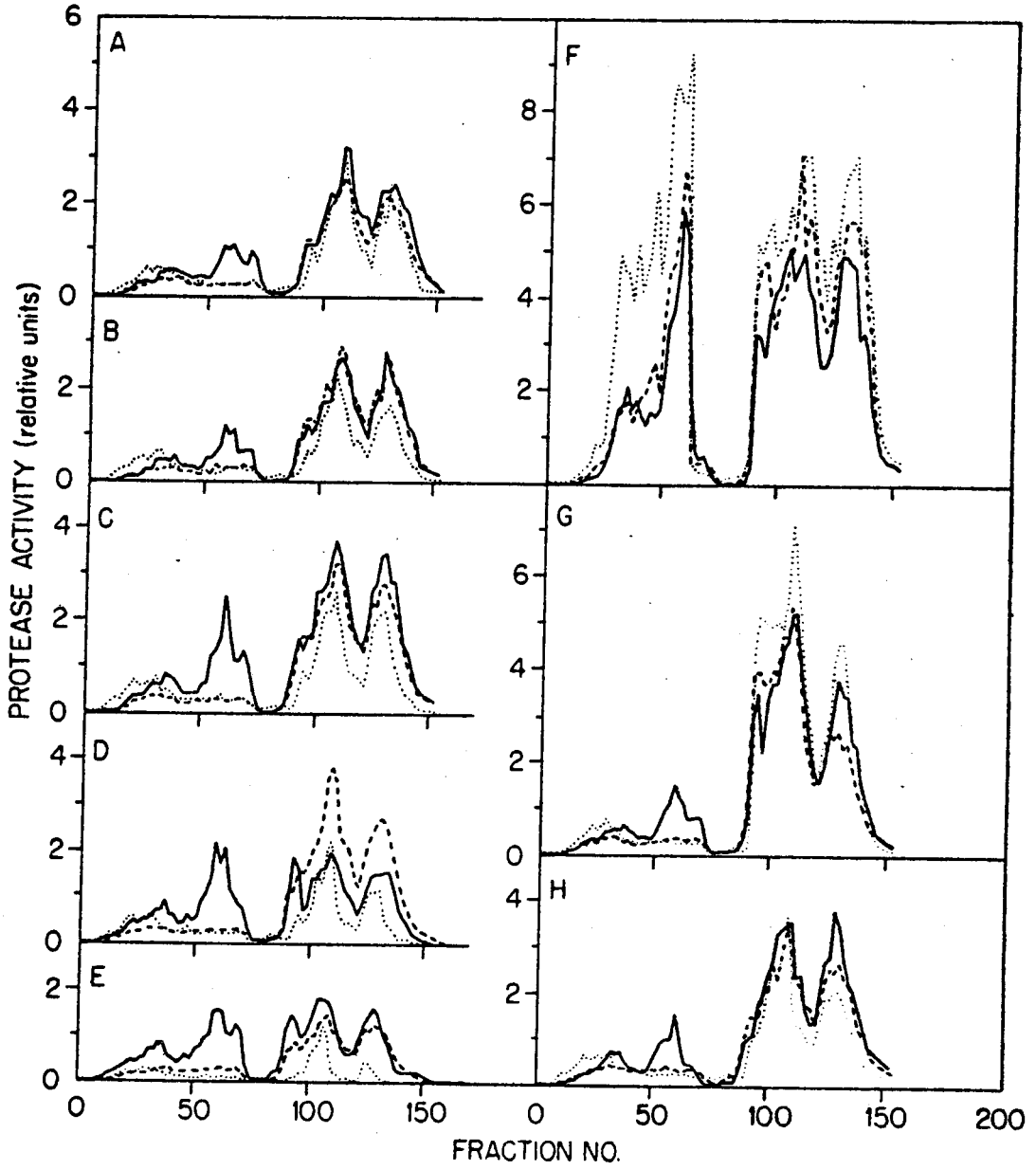
A
B
C
D
E
F
G
H
PROTEASE ACTIVITY (relative units)
FRACTION NO.
0
50
100
150
200

Fig. 4.3.

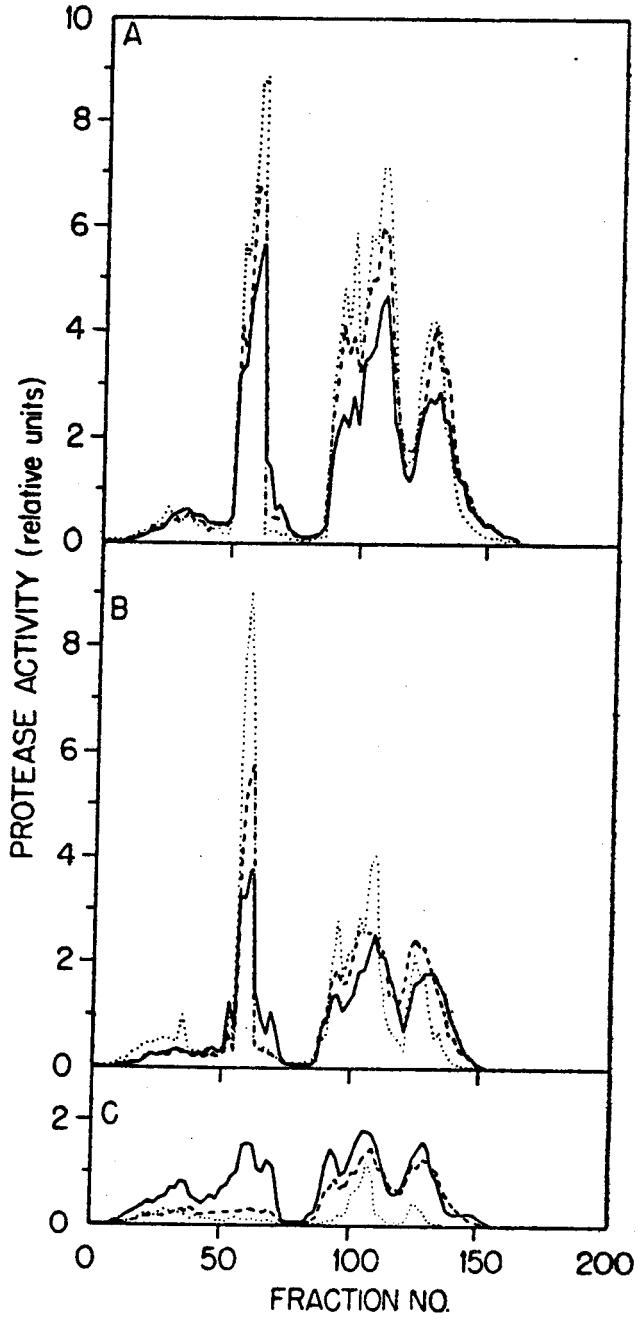

Fig. 4.4. Release of proteolytic activities from an early logarithmic growth phase of N. crassa during slow freeze and storage at -15°C. Ammonium sulfate fractionation and DEAE cellulose chromatography were done as shown in Fig. 4.1. The chromatograms were assayed for proteolytic activity with azocoll at pH 8.5 (····), pH 7.0 (----), and pH 5.0 (——). Panel A shows the proteolytic activity present after three slow freeze (-15°C)/thaw (25°C) cycles. Panel B shows the proteolytic activity present after one slow freeze (-15°C)/ thaw (25°C) cycle. Panel C shows the proteolytic activity assayable immediately after chromatography.

and cryptic behavior. A similar multiplicity of intracellular proteases in N. crassa also has been recently observed by Tan and Marzluf (53). In this report we have examined the influence of storage conditions on the cryptic behavior of proteases from N. crassa grown to an early logarithmic stage of growth. The data indicate that there are substantially more proteases in the 0-40% ammonium sulfate fraction than we saw earlier. We did not, for instance, note the activity contained in the first 70 fractions, since much of this activity was released only after storage for a minimum of nine days at 4°C (see Fig. 4.1). The release of these activities presumably is a consequence of the degradation of natural inhibitor and/or the activation of zymogens.

Rapid freezing at -196°C and subsequent storage at -20°C offers a reliable method of limiting the expression of the proteases to the activity present at the time of freezing (see Fig. 4.3). This has an obvious practical application and would be helpful to the biochemist concerned with proteolytic artifacts. On the other hand, storage conditions which allow for a slower freezing rate, such as freezing in a relatively large volume in a plastic container at -15°C, offer pitfalls to the investigator. For example, Fig. 4.4 shows that substantial amounts of activity are released under these conditions. We found that nearly one-third of our fractions were unfrozen after 23 hours in storage, a situation that allows for the expression of cryptic proteolytic activity.

Figure 4.1 demonstrates that, eventually, nearly every chromatographic fraction displays proteolytic activity. In a typical protein

purification, a number of fractionation steps are carried out over a three to seven day period. Hence it is likely that in N. crassa, and in other organisms as well, many proteins could be attacked by various proteases during a given purification procedure. Pringle (38) has already forewarned investigators of the possibility of proteolytic artifacts by pointing out that some 40 "purified" yeast enzymes which he examined contained trace amounts of proteolytic activity.

In our previous report (20) we were surprised by the appearance of so many proteolytic activities in N. crassa. In the present report we give evidence that there may be still more proteases. We suspect that further investigation will reveal a highly complex array of proteases whose functions, though unknown at this time, are likely involved in metabolic regulation.

CHAPTER V

A PLASTIC PIPETTE TIP ARTIFACT: AFFINITY FOR PROTEASES

Summary

Four enzymes were found to bind plastic pipette tips and subsequently leach out during serial dilution. Thermolysin and subtilisn were detectable in the wash from the pipette tips after more than 20 dilutions. Trypsin and chymotrypsin were also found to bind and subsequently leach out. Subtilisn was found to bind to three common laboratory containers: a plastic scintillation vial, a plastic centrifuge tube, and a plastic freezing vial. The ability to bind these enzymes is heat-labile, and the pipetting artifact can be moderated by autoclaving the plastic tips prior to use.

Introduction

In an earlier study (47) we used several commercial proteases to examine the sensitivity of a proteolytic assay in which azocoll was the substrate. We later found that one protease, subtilisn, was exceptionally reactive in this assay, but apparently could not be diluted out. The cause of this behavior is the affinity of plastic for proteases; several proteases bind to plastic pipette tips and common plastic containers and can subsequently leach out during sequential dilutions or rinses with buffer.

Materials and Methods

Azocoll assay

Assays for proteolytic activity, using azocoll as the substrate, were carried out as previously described (47).

Dilutions

Unless otherwise noted, ordinary 1:10 serial dilutions of enzyme were made. For "mock" dilutions, 100 µl of stock enzyme solution (normally at a concentration of 1 mg/ml) were drawn into a plastic pipette tip, then released. After this procedure was repeated twice, the empty pipette tip was used to make 1:10 serial dilutions with buffer only. Trypsin and chymotrypsin were buffered in 0.1 M potassium phosphate, pH 8.5; subtilisn in 0.1 M potassium phosphate, pH 7.0; and thermolysin in 0.02 M Tris-acetate, pH 6.0, and 2 mM $CaCl_2$.

Materials

Azocoll was purchased from Calbiochem. Trypsin, chymotrypsin, subtilisn, and thermolysin were purchased from Sigma. Disposable 200 µl plastic pipette tips (used with automatic pipettors) were purchased from Rainin Instruments, Markson Scientific Co., or Bio-Rad. All other chemicals used were reagent grade.

Results

We first noticed an anomaly in the behavior of subtilisn when we conducted simple concentration-dependent proteolytic assays in which azocoll was the substrate. Subtilisn's activity could not be diluted out; i.e., we could detect proteolytic activity when, in theory, only

a few molecules of protease should have been present. Further assays produced similar results for other proteases, most notably for thermolysin. By a process of elimination, we soon associated this behavior with the plastic pipette tips we were using to make serial dilutions, and found that the behavior could be moderated by use of sterile pipette tips.

Figure 5.1A shows the salient features of the dilution anomaly. When a 1:10 serial dilution is made with a single nonsterile pipette tip, beginning with an initial 1 mg/ml concentration of thermolysin, near-maximal activity is detected at 10^{-11} mg/ml enzyme. When further dilutions are made, this time with a sterile pipette tip, the activity falls off rapidly. If the original nonsterile tip is used to make more dilutions, the activity returns. The experiment demonstrates that thermolysin binds to nonsterile pipette tips and subsequently leaches out during dilutions.

This behavior is perhaps better displayed when mock dilutions (see Methods and Materials) are made, where the only source of protease is that bound to the pipette tip. Figure 5.1B shows that a nonsterile pipette tip releases a considerable amount of thermolysin even after 12 mock dilutions. Use of a sterile pipette tip moderates, but does not eliminate, this release. Similar experiments using the commercial proteases trypsin, chymotrypsin, and subtilisn give similar results (see Fig. 5.3).

If a given percentage of the protease present binds to a pipette tip, then a reduction in the initial amount of protease should reduce the amount of protease available to both bind to and leach from the

Fig. 5.1. Protease binding during serial dilution. A nonsterile pipette tip was used to make 1:10 serial dilutions of a 1 mg/ml stock solution of thermolysin (A). After 11 dilutions, a sterile tip was used to make six more dilutions; then the original nonsterile tip was used to make the final six dilutions. All dilutions were assayed for proteolytic activity with azocoll. Mock dilutions of thermolysin (see Methods and Materials), with a sterile (●) and a nonsterile (○) pipette tip, are shown in B. In each case the initial concentration of thermolysin was 1 mg/ml; dilutions were assayed for proteolytic activity with azocoll.

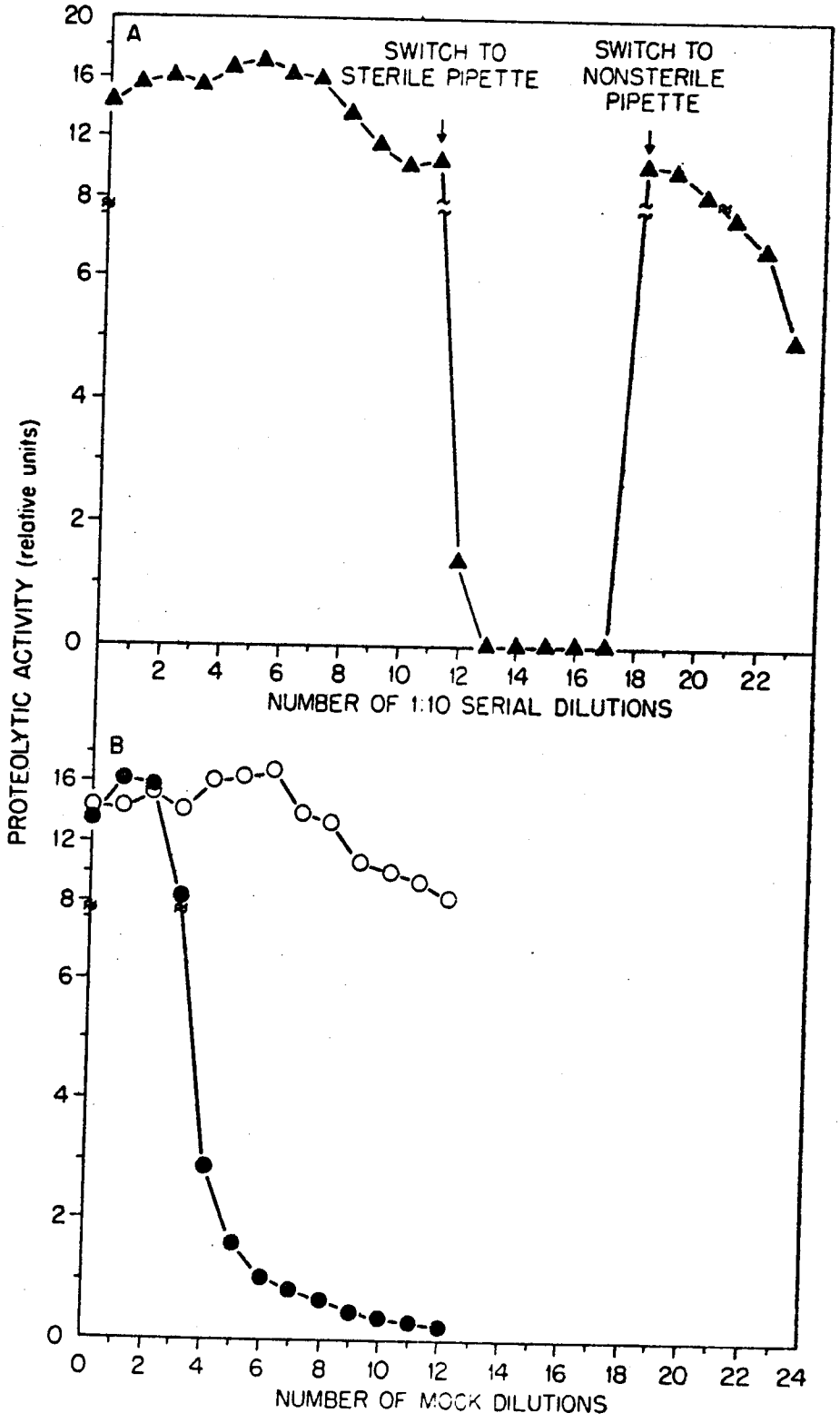
A
SWITCH TO STERILE PIPETTE
SWITCH TO NONSTERILE PIPETTE
NUMBER OF 1:10 SERIAL DILUTIONS
B
PROTEOLYTIC ACTIVITY (relative units)
NUMBER OF MOCK DILUTIONS

Fig. 5.1.

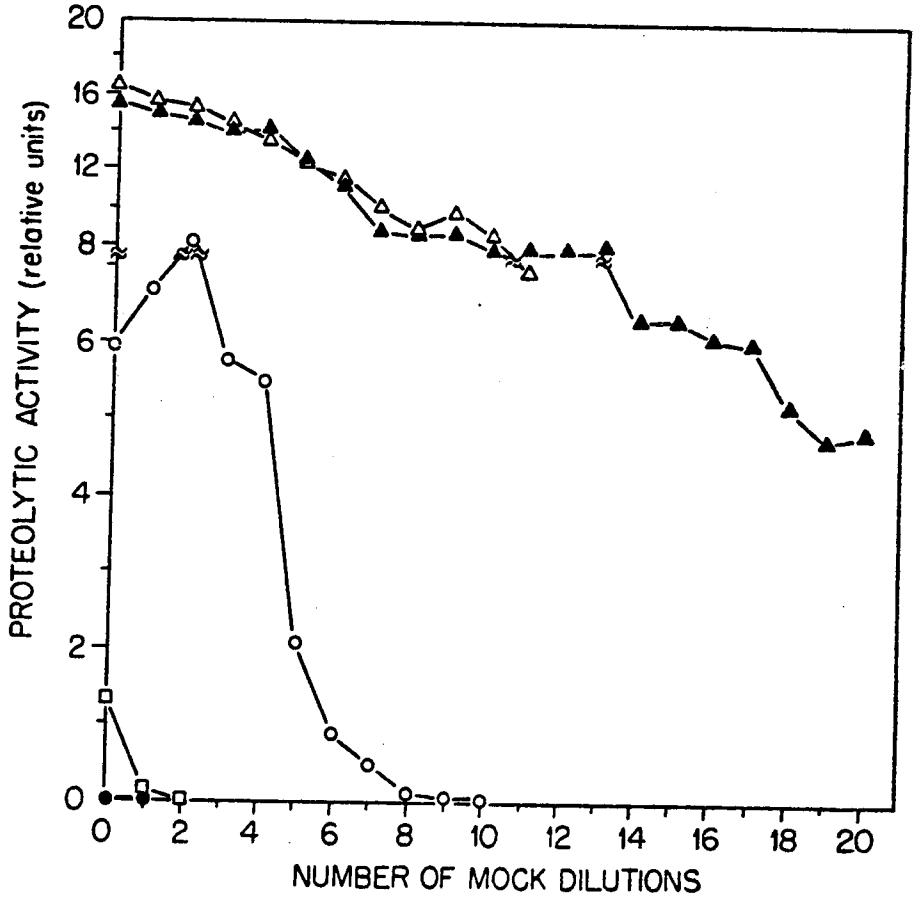

Fig. 5.2. Effect of initial concentration on protease binding to and release from plastic pipette tips. Different initial concentrations of thermolysin (▲, 1 mg/ml; △, 0.1 mg/ml; ○, 0.01 mg/ml; □, 1 µg/ml; and ●, 0.1 µg/ml) were used to make mock dilutions (see Materials and Methods). The dilutions were then assayed for proteolytic activity with azocoll.

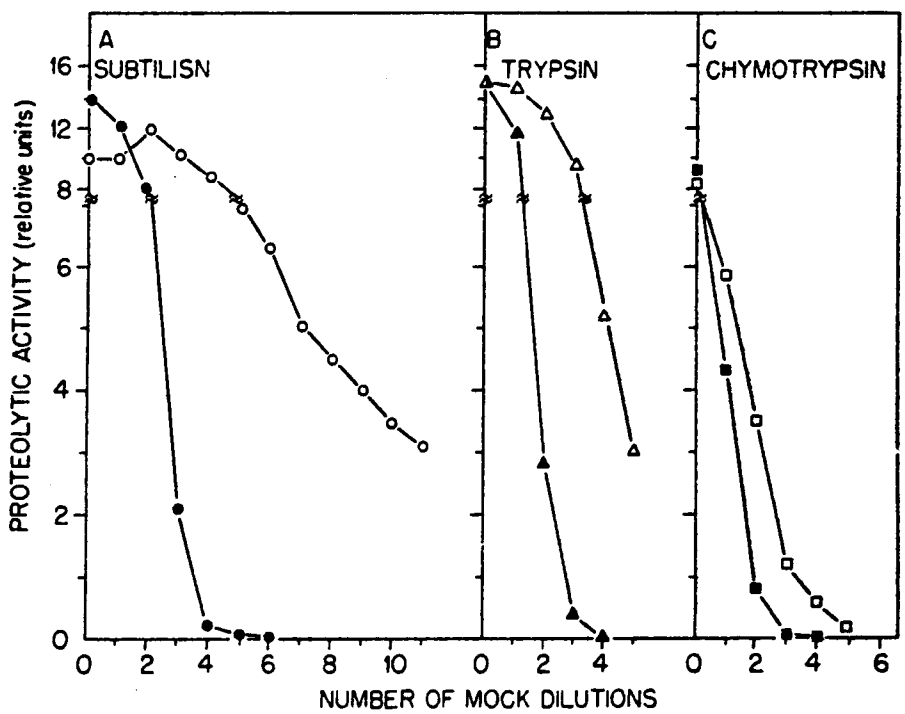

Fig. 5.3. Protease binding and release during mock dilution. Mock dilutions (see Materials and Methods) of subtilisn made with a sterile (●) and a nonsterile (○) pipette tip, are shown in A. Mock dilutions of trypsin made with a sterile (▲) and a nonsterile (△) pipette tip, are shown in B. Mock dilutions of chymotrypsin made with a sterile (■) and a nonsterile (□) pipette tip, are shown in C. Initial concentration of each protease was 1 mg/ml; dilutions were assayed for proteolytic activity with azocoll.

pipette tip. Figure 5.2 shows results obtained when the initial concentration of thermolysin is reduced from 1 mg/ml to 0.1 µg/ml, followed by mock dilutions. Somewhere between 1 µg/ml and 0.1 µg/ml a threshold is reached, below which no thermolysin binding is detectable within the limits of our assay. Note also that thermolysin activity is readily assayable after 20 mock dilutions; the same is true for subtilisn (data not shown).

We examined other plastic containers commonly found in a biochemistry laboratory. In each case the procedure was to introduce a 1 mg/ml solution of subtilisn, remove it, rinse the container with buffer, remove the buffer, and assay the buffer rinse for proteolytic activity with azocoll. Figure 5.4 shows that a plastic scintillation vial, a plastic centrifuge tube, and a plastic freezing vial (used to store samples in liquid nitrogen) all have the ability to bind subtilisn and release it during several buffer rinses.

Discussion

We found four commercial proteases that bind to pipette tips: thermolysin, subtilisn, trypsin, and chymotrypsin (see Figs. 5.1 and 5.3). Although the amount of proteolytic activity released during mock dilutions differs from protease to protease, the use of sterile pipette tips always results in a reduced amount of activity detectable. It is not clear whether sterilization of the pipette tip inhibits the initial binding of the protease or its subsequent leaching from the pipette tip; either effect would result in a reduced amount of detectable proteolytic activity. The process of autoclaving does

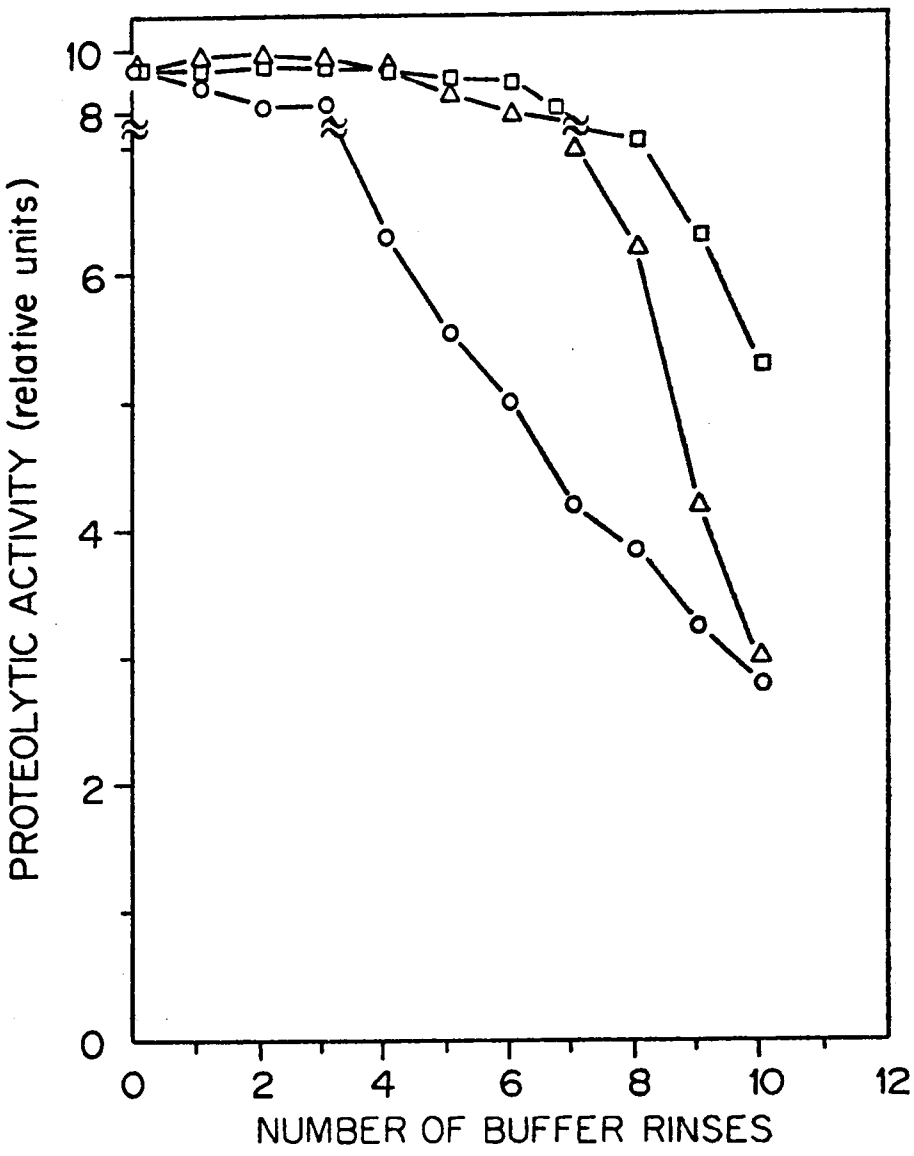

Fig. 5.4. Subtilisn binding to and release from plastic containers. One ml of subtilisn (1 mg/ml) was introduced into a plastic scintillation vial (□), a plastic centrifuge tube (○), and a plastic freezing vial (△). The enzyme solution was removed, then each container was rinsed 11 times with 0.5 ml of buffer (buffer was changed for each rinse). The buffer rinses were assayed for proteolytic activity with azocoll.

produce a hydrophobic surface on which small droplets of solution cling during a serial dilution. This may account for some of the activity that persi ts with mock dilutions even when sterile tips are used. We also found several proteases (pepsin, carboxypeptidase B, and leucine amino-peptidase) that did not release proteolytic activity during mock dilutions. However, because each of these proteases displays low proteolytic activity in our assay, binding cannot be ruled out.

Each protease that is readily assayable in our system also binds, in varying degrees, to plastic. We found that three different brands of pipette tips all bind protease approximately equally (data not shown). The problem for the biochemist is primarily practical, i.e. how does one limit this behavior? We have found the sterilization of pipette tips helps to control protease binding. Use of pipette tips only once during serial dilutions or other repetitive pipetting procedures would also avoid contamination. Perhaps the best approach, as Pringle has stated (38, 39), is to be continually aware of the difficulties which unwanted and unheeded proteolysis can bring the researcher.

CHAPTER VI

EVIDENCE FOR MULTIPLE PEPTIDASES IN *NEUROSPORA CRASSA*

Summary

An early logarithmic growth phase culture of *Neurospora crassa* was fractionated with ammonium sulfate, chromatographed with DEAE cellulose, and assayed for intracellular proteolytic activity with the general substrate azocoll. Chromatographic fractions were then electrophoresed on 7% polyacrylamide slab gels and assayed for peptidase activity, using an *in situ* staining procedure which reveals peptide digestion as a sharp blue band. Different peptides produced different banding patterns, which were then compared and tabulated. We found eighteen peptidases distinguishable from one another on the basis of substrate specificity. Furthermore, in many instances the chromatographic location of peptidase activities did not correlate with the location of proteolytic activities as revealed with the azocoll assay.

Introduction

Until recently, intracellular proteases were thought to be located primarily in the lysosome (or in the analagous plant vacuole) and to be relatively few in number (23, 31, 43). Now, however, the number of proteases recognized within particular organisms is steadily increasing. For instance, in 1975 it was reported that *Saccharomyces cerevisiae* contained four proteases (23), but a more recent report assigns seven specific proteases to this organism (56).

Similarly, the number of proteases reported in Neurospora crassa increased from three (23) to five (46) before we used azocoll as a general substrate to examine the proteolytic activity of chromatographic fractions from several N. crassa ammonium sulfate precipitations (20), and found evidence for at least twenty five intracellular proteases. However, the lack of substrate specificities in our study limited positive identification of the presumed multiple proteases. Tan and Marzluf (53) devised an efficient staining method to detect specific peptidase activity on polyacrylamide gels, and described eleven distinct peptidases in total soluble proteins from N. crassa. In this study, we combined Tan and Marzluf's electrophoretic assay (modified for slab gels) with DEAE cellulose chromatography and detected eighteen distinct peptidases. Moreover, we anticipated correlation between the azocoll-reactive and the peptide-reactive fractions from DEAE chromatography, but in fact the latter represented only a portion of the intracellular proteolytic activity indicated by digestion of azocoll.

Materials and Methods

Growth of organism

N. crassa was grown as described earlier (19) and harvested at an early logarithmic growth stage (7 g/l wet weight). The mycelia were lyophilized, ground in a Wiley mill, and stored at -20°C.

Protein fractionation and chromatography

Soluble proteins were fractionated as described earlier (48). A 0-45% ammonium sulfate fraction was prepared from lyophilized

mycelia, desalted on Sephadex G-25, and chromatographed on DEAE cellulose. After chromatography the fractions were stored at -25°C.

Azocoll assay

The chromatographic fractions were assayed for proteolytic activity with azocoll as described earlier (47).

Peptidase assay

Gel electrophoresis of the chromatographic fractions was done according to a modification of the procedure by Tan and Marzluf (53). 100 X 140 X 1.5 mm polyacrylamide slab gels (7%) were cast with L-amino acid oxidase (0.67 mg enzyme per ml of gel solution) becoming immobilized within the gel. The upper and lower buffer chambers contained 0.005 M Tris - 0.040 M glycine, pH 8.3. The chromatographic fractions (30 µl), containing sucrose and bromophenol blue, were layered onto the gel surface and electrophoresed at 130 mA for three hours. The gels were stained for peptidase activity by incubating in a solution of 170 µg of peptide, 170 µg of Nitro Blue Tetrazolium, and 17 µg of phenazine methosulfate per ml of 0.1 M Tris-HCl buffer, pH 7.0, at 37°C. Gels were incubated for approximately 18 hours to visualize all peptidases reactive with the peptide substrate employed.

Chemicals

Azocoll was purchased from Calbiochem. DEAE cellulose Type 20 was purchased from Brown Paper Co. Phenazine methosulfate, L-amino acid oxidase, and Nitro Blue Tetrazolium were purchased from Sigma. All other chemicals used were reagent grade.

Results

The fresh DEAE cellulose chromatography fractions were assayed for initial proteolytic activity towards the substrate azocoll (Fig. 6.1). Each fraction was assayed for the presence of acidic (pH 5.0), neutral (pH 7.0), and alkaline (pH 8.5) proteolytic activity. The high amount of acidic activity relative to the neutral and alkaline activities is common for fresh preparations (20).

The chromatographic fractions were next layered on 7% polyacrylamide slab gels to separate any peptidases present. The staining technique employed reveals those peptidases reactive towards the peptide substrate as sharp blue bands within the gel. Many of these bands are readily visable within thirty minutes incubation, while other peptidases require overnight incubation for the reaction to be detected. A comparative example of two gels, one incubated with prolyl-phenylalanine and the other with glycyl-methionine, is shown in Fig. 6.2, A and B, respectively. Note that the banding patterns of the two gels are distinctly different. By using a broad spectrum of peptide substrates, we obtained several banding patterns, each distinctive for the particular substrate tested. Comparison of the patterns reveals substrate-specific peptidases, which we have tabulated in Table 6.1.

Discussion

In an earlier report (20) we presented evidence that suggests *N. crassa* contains multiple proteases. The idea of a multiplicity of proteases has been recently confirmed by Tan and Marzluf (53), who

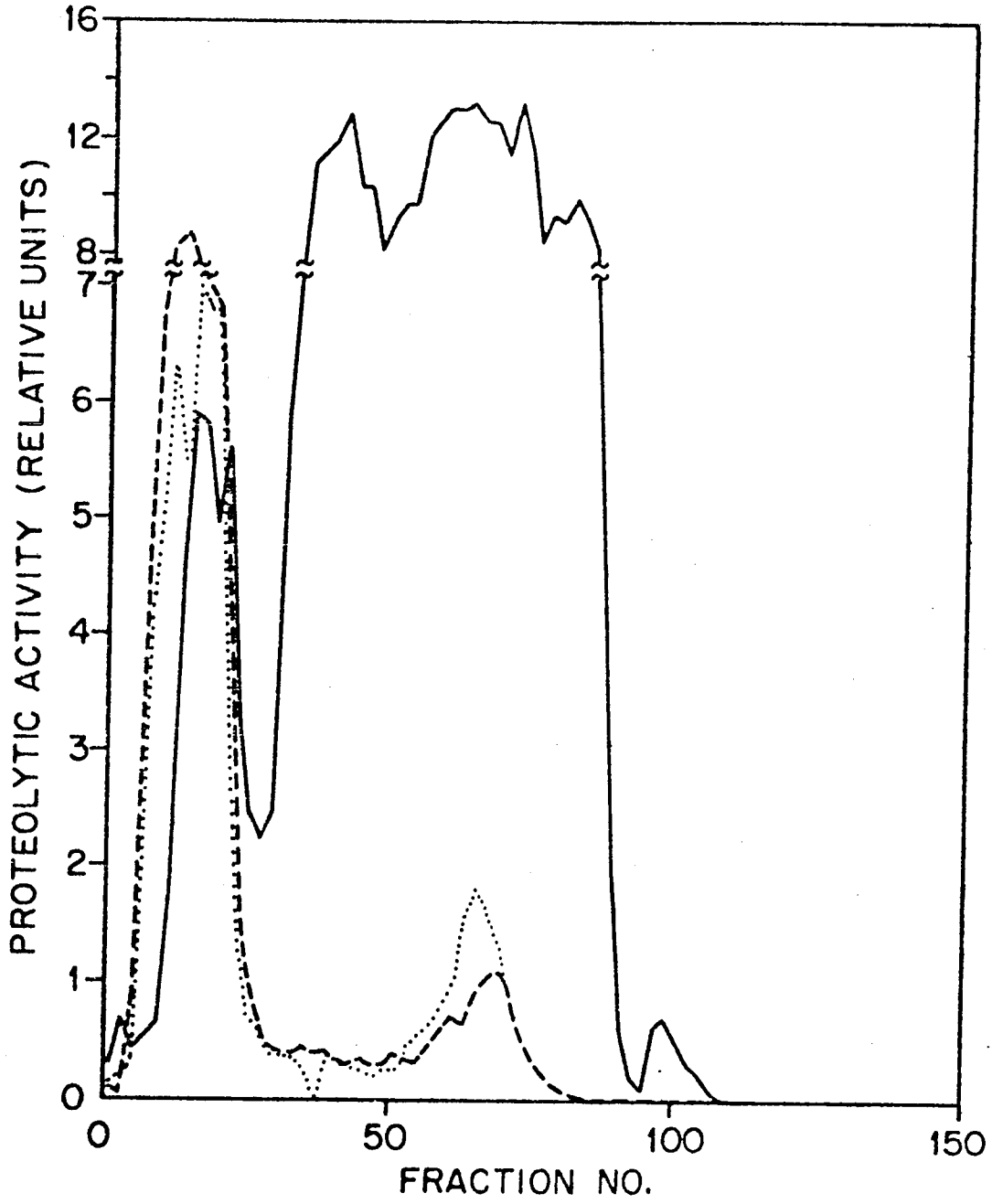

Fig. 6.1. Initial proteolytic activity in fractions from early logarithmic growth phase N. crassa. A 0-45% ammonium sulfate fraction was chromatographed on DEAE cellulose as described in Materials and Methods. The chromatographs were assayed for proteolytic activity with azocoll at pH 8.5 (····), pH 7.0 (----), and pH 5.0 (——).

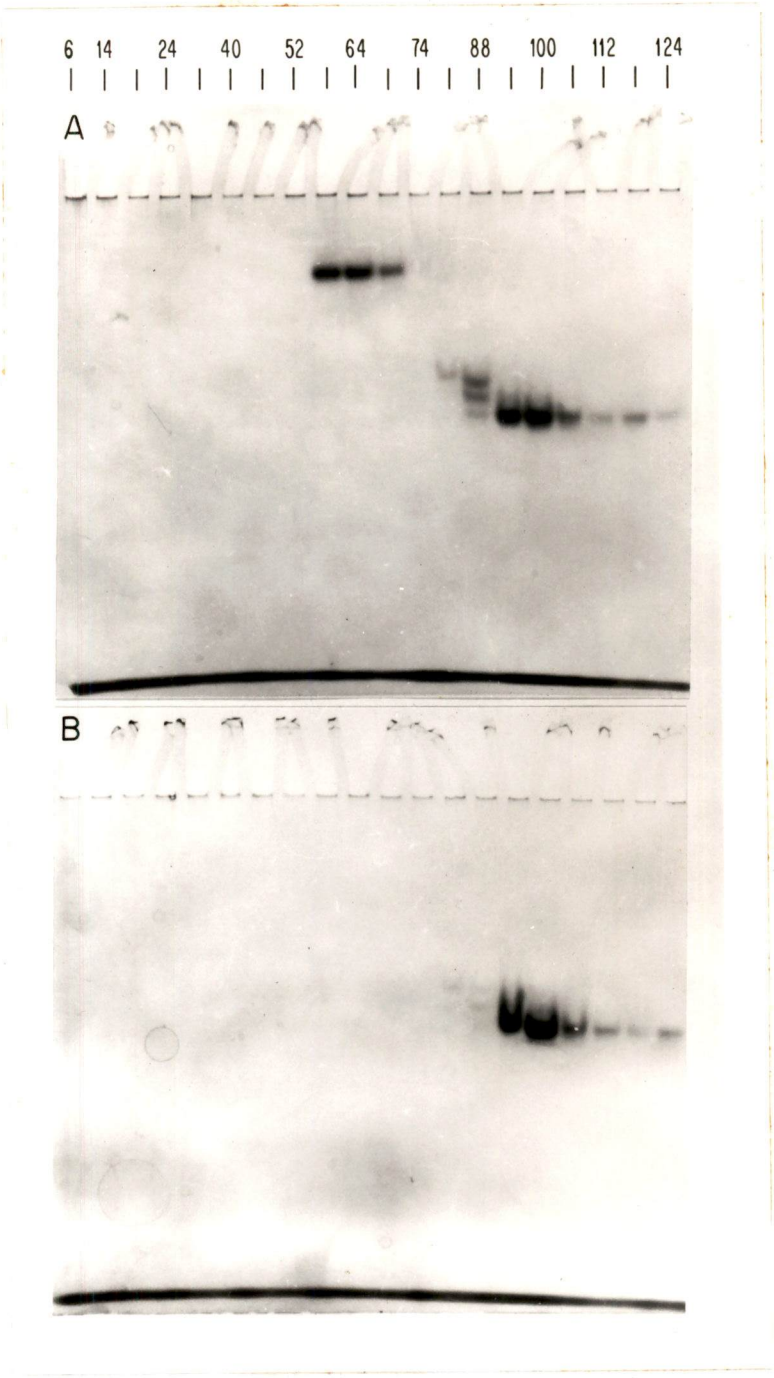

Fig. 6.2. (A) pro-phe and (B) gly-met reactive peptidases of N. crassa. DEAE cellulose column fractions (column fraction numbers listed at top of figure) were electrophoresed on 7% polyacrylamide slab gels, and the gel was stained for peptidases reactive to the dipeptides pro-phe and gly-met (see Materials and Methods). Each reactive peptide resulted in a distinctive banding pattern, as represented here.

TABLE 6.1

Multiple Peptidases Revealed by Polyacrylamide Slab Gel Electrophoresis

Peptidase Species	DEAE cellulose Fraction No.	R_f	Comparative Peptide Reactivity
1	40	.47	+ (val-tyr)
2	58-70*	.16	+ (pro-phe; val-tyr)
3	58-76	.39	+ (met-ala; leu-tyr; leu-phe; val-tyr) - (leu-gly)
4	58-76	.43	+ (leu-tyr) - (met-ala; leu-phe; val-tyr; leu-gly)
5	58-76	.46	+ (leu-phe; val-tyr; leu-gly) - (met-ala; leu-tyr)
6	82	.39	+ (g;y-met) - (leu-leu-leu)
7	82	.43	+ (gly-met) - (leu-leu-leu)
8	88	.39	+ (leu-leu-leu)
9	88-94	.19	+ (tyr-gly-gly)
10	94	.43	+ (leu-leu-leu) - (gly-met)
11	94-100	.04	+ (leu-tyr; leu-phe)
12	94-100	.06	+ (leu-tyr; leu-phe)
13	94-106	.46	+ (poly-arg; gly-asp. acid; his-phe)
14	100-112	.48	+ (leu-leu-leu)
15	112-124	.43	+ (met-ala) - (leu-tyr)
16	112-124	.46	+ (met-ala; leu-phe) - (gly-asp. acid; poly-arg)
17	136	.42	+ (val-tyr; leu-tyr)
18	148-160	.44	+ (leu-tyr; phe-tyr)

found eleven peptidases, each of which displays a particular and distinctive pattern of substrate specificity. Their system used gel electrophoresis (tube) to separate total soluble proteins prior to *in situ* staining for peptidase activity.

Through suitable modification for slab gel electrophoresis, we used this assay to examine DEAE cellulose chromatography fractions from a 0-45% ammonium sulfate preparation of soluble proteins. We tested more than thirty peptide substrates, fourteen of which elicited positive reactions with the *N. crassa* peptidases. Each substrate reveals a different and distinct banding pattern (Fig. 6.2A and 6.2B). By comparing the patterns, we have identified eighteen peptidases (Table 6.1) present in the protein fraction. We originally assumed that many of the peptidases, perhaps all of them, would correlate with proteolytic activities revealed by the azocoll assay (Fig. 6.1). Comparison of Figs. 6.1 and 6.2 shows this not to be the case, that in fact many of the peptidases are not found in the area of the DEAE column which contains proteolytic activity assayable with azocoll. This substantiates our earlier suspicion that *N. crassa* has many more intracellular proteases than heretofore recognized. It likewise suggests that, as other assays for the detection of multiple proteases are developed, more proteases are likely to be found in *Neurospora*.

It is now clear that some organisms possess multiple proteolytic activities. The concept of multiple intracellular proteases implies that proteases may perform more roles in the regulation of cellular biochemistry than presently envisioned. The established functions include maturation of nascent proteins, hydrolysis of defective and/or

cellular proteins in the turnover process, and breakdown of extracellular proteins as a source of cellular nutrition (53). As the number of known intracellular proteases and peptidases increases, the possibilities of other regulatory functions for them likewise increases.

CHAPTER VIII

CONCLUDING REMARKS

The azocoll Protease Assay

Of fundamental importance to a research plan is the assay. The assay should be reproducible, quantitative, and measure what the researcher intends to measure. Chapter II deals primarily with the mechanics of the azocoll assay, which was used to obtain most of the data presented here.

Azocoll is insoluble in aqueous solution. Paradoxically, this property makes it both a highly desirable substrate and yet a very difficult one with which to work. The desirability stems from the fact that, short of some form of proteolytic attack, there is no avenue for the material to solubilize and subsequently be detected spectroscopically. Therefore, there is virtually no background to the reaction and the researcher is assured that the assay is measuring what is intended. However, the same property of aqueous insolubility normally restricts usage of azocoll to a limited number of individual assays. This is because dry azocoll is difficult to aliquot in the small quantities needed in each reaction mixture. The researcher is usually resigned to weighing each 10-20 mg aliquot on a balance, a tedious and time-consuming task.

The dispensing machine described in Chapter II (Figs. 2.1 and 2.2, pg 9, 10) circumvents this problem. The machine was designed to quantitatively and reproducibly deliver small aliquots of azocoll. The dispensing machine also delivers twelve aliquots simultaneously.

These features allow the researcher to aliquot azocoll quickly and efficiently in reaction mixture test tubes. This in turn makes feasible large-scale survey experiments, where hundreds of assays are necessary to measure the proteolytic activity. Thus the dispensing machine solves a technical problem and is perhaps most responsible for the successful completion of the many azocoll assay experiments described in the preceeding chapters.

Chapter II also mentions one other important characteristic, which should be underscored, of the azocoll assay. There are several protease assays currently available to investigators. Some of these, such as casein or hemoglobin digestion (27, 2), have been used for several decades and are recognized as standards. Other assays monitor loss of specific enzymatic activity (25, 26, 30, 44) or utilize relatively recent techniques, such as radio-labeled amino acid uptake (8, 57) or gel electrophoresis (1, 53). Despite the diversity of approach to measuring proteolysis, many of the current assays are completed within a short period of time, normally 30-60 minutes. If the assay is not sensitive enough, due perhaps to a low level of protease or a relatively unreactive substrate for the protease being tested, proteolytic activity could go undetected. The azocoll assay, as we have modified it, allows a standard reaction time of 10 hours, and thereby increases the sensitivity of the assay to detection of picomolar quantities of several commercial proteases. This, combined with the rapid aliquoting technique, allowed us to effectively screen large numbers of column fractions and detect the proteolytic activity present.

Experimental Results

A fortuitous event at the beginning of this research had a significant bearing upon the choice of further experiments. The initial column fractions were stored in a walk-in freezer (-10°C) for several months while a series of commercial inhibitors were examined for their effects upon the expressed proteolytic activities of the column fractions. After six months these stored fractions were re-assayed to establish base-line proteolytic activity at that time. Rather than the expected decrease and/or stable levels in activity, the result was a surprising increase in the proteolytic activity of nearly every column fraction (Figs. 3.3 and 3.4, pg 24, 27). This led to the series of experiments described in Chapter IV and the discovery that the major Neurospora proteolytic activities are cryptic in nature. The increase in proteolytic activity in column fractions can be nicely monitored by storage at 4°C and periodic assaying (Fig. 4.1, pg 50).

The data presented here supports the hypothesis that there is a multiplicity of proteases in N. crassa. That the origin of the proteolytic activity is not microbial was shown in Chapter IV. The pattern of activity is not generated from faulty chromatographic conditions, as marker enzymes show typical separation. Rerunning fractions containing proteolytic activity shows this activity runs true with the same chromatographic R_f value. A reasonable interpretation is that the proteolytic activities are derived from Neurospora and are not generated through poor chromatographic technique.

There is ambiguity, however, on the question of whether or not the expressed proteolytic activities do indeed represent separate and distinct proteases. It is possible that only a few proteases are complexed with inhibitors and other protein moities (as a result of cellular disruption) and are subsequently differentially bound to the DEAE cellulose columns. This would result in the appearance of a far greater number of endogenous proteases than actually exist under cellular conditions. It is also possible that the expressed activities are the result of active breakdown products of a few intracellular proteases. However, Tan and Marzluf (53) describe eleven N. crassa peptidases which display specficity towards different peptide substrates, and our slab gel studies, a modification of their technique, show several peptidases which also are specific in their peptide hydrolysis abilities (Table 6.1, pg 75). Many of the peptidase activities revealed by the gel studies do not correspond chromatographically to proteolytic activities expressed in the azocoll assay, which suggests that other assays could be employed to demonstrate specificity of the azocoll activities. These specific peptidases already revealed by electrophoresis techniques argue against the protein-protease complex and autolysis theories advanced to explain the observed multiplicity of proteolytic activities. Though either a protease capable of complexing with several different protein species or a protease having undergone autolytic digestion might be capable of binding differentially to a DEAE cellulose column, it is not likely that the specificity of such a protease would be altered.

Implications

In the past decade proteases have become recognized for the diversity of functions they perform within cells (5, 14, 23). However, most investigators still cling to the notion that proteases reside in lysosomes (or the analagous vacuoles of plants) and primarily function to degrade bulk protein into constituent amino acid residues for use by the protein synthetic apparatus (42). While this idea certainly has not been disproven, there are some complications which necessarily evolve from such a format. Blobel (9) has pointed out that it is difficult to account for the great diversity of protein turn-over rates commonly found in a cell, especially if the protein must first be transferred in some fashion to the lysosome. Some type of differential process, such as differing rates of access to the lysosome by the substrate proteins or varying rates of hydrolysis once inside the lysosome, must be envisioned to explain turn-over rates. Moreover, the cell must be capable of recognizing those enzymes in the cytosol which need to be rapidly degraded. Consequently, the cell would have to maintain a type of rapid transport system to shuttle proteins to and from the lysosomes. Finally, lysosomes maintain a relatively low pH, whereas many proteases recently discovered have a pH optimum near neutral (13, 45, 53, 57). All of this argues that the lysosome is probably not the only realm of proteolytic activity within the cell.

Proteases are often discovered by accident. For instance, many investigators have reported proteolytic contaminants within their particular enzyme system (21, 29, 45, 49, 50). Though not necessarily stated in their report, it is relatively easy to envision that

investigators first monitor protein changes in their *in vivo* or *in vitro* systems, then search for the protease responsible. In addition, those investigators who do concentrate on the study of proteases often choose extracellular, or other easily obtainable, proteases for their experiments. This is especially true in microorganisms (23). The intracellular proteases have not been as extensively studied, though, and the reasons for this are many. In cell extracts, intracellular proteases often occur in an inactive form, either as a zymogen or in combination with specific inhibitors. Successful assay of these forms is difficult, especially with the more conventional techniques. Intracellular proteases frequently display a narrow specificity as well, rendering many of the common substrates unsuitable for their detection.

The type of screening techniques necessary to detect intracellular proteolytic activities have only recently been developed. These techniques are now being applied, and multiple proteases have been reported in *N. crassa* (20, 53), *Salmonella* (12), *Dictoselium discoideum* (35), and *E. coli* (32). However, as the number of intracellular proteases detected within a given organism grows, then some attention must be given to their possible roles within the cell. Some of these roles, such as involvement in protein maturation (45), differentiation and development (44), or degradation of nonsense proteins (28, 43) have been well documented. But in each case the implication is that this cellular function is highly specialized and limited in scope. If the cell contains relatively few proteases, such as the

seven proteases found thus far in yeast (56), it is reasonable to argue that these enzymes have a rather restricted function in the control of cellular biochistry. But if, as the data presented here suggests, organisms can and do contain a broad spectrum of proteases and peptidases, it now becomes reasonable to assume their involvement in biochemical control processes is much more extensive than earlier envisioned. The isolated cases of proteases being necessary for certain differentiation processes or protein maturation, to give but two examples, may in fact represent a common and effective method whereby the cell regulates its biochemical machinery. At the very least, it is perhaps naive to view protein synthesis as a wondrous and complicated process, while protein degradation is relegated to a simple, somewhat pedestrian role by comparison.

It is not our contention that the multiple proteolytic activities expressed in the chromatographic data presented here are in fact separate and distinct proteases. While this interpretation remains a probable one, especially when the results of the gel studies are included in the analysis, much work needs to be done to clearly establish such a relationship. However, there is one aspect of the data which simply cannot be denied. After an appropriate incubation period, there are virtually no chromatographic fractions devoid of proteolytic activity. This fact has clear implications with respect to general protein research, especially in N. crassa.

Pringle has noted that "Proteolytic artifacts are pervasive, perplexing, persistent, and pernicious" (38). He compiled a list of 45 yeast proteins known to have undergone proteolytic modification,

even after extensive purification procedures (38). While proteolytic modification can result in inactivation of a protein of interest, often the modification(s) is more subtle. The modifications may not be reflected by a reduction or disappearance of enzymatic activity, but rather in the appearance of apparent isoenzymes or an alteration in the kinetic parameters of the protein of interest. In any event, proteolytic contamination is unlike other forms of contamination, which merely require the investigator to more rigorously purify the protein of interest. Contamination by a protease can, and likely does more often than recognized, lead to *in vitro* alteration. This in turn reduces the biological relevance of the protein being investigated.

While the enzymatic behavior of a protease has an acknowledged subtlety, the physical behavior may be subtle as well. Chapter V describes the specific binding, and subsequent leaching, activities of several commercial proteases to common plastic items. This phenomenon was certainly unexpected, but it suggests a pathway for proteolytic contamination of other protein species. Purification procedures routinely employ plastic containers, many of which are rinsed briefly and reused. Column fractions are often collected and then stored in plastic containers. The practical result is that the binding of proteases to plastic represents another obstacle to the unsuspecting investigator.

The fact that there is so much proteolytic contamination in the column fractions from our *N. crassa* protein extracts suggests that it is difficult to purify a protein, especially from *Neurospora*, and be

absolutely sure that the protein is identical to the *in vivo* species. The cryptic nature of most of the proteolytic activities only magnifies the problem, because this lulls the investigator into thinking the potential hazards are less than they really are. The value of this dissertation lies partly in recognizing the size and scope of the problems of proteolytic contamination, particularly as they apply to protein research with *N. crassa*.

Conclusion

With the more recent discoveries of proteolytic functions has come an appreciation of the diversity and subtlety of the roles that proteases perform within the cell. Moreover, because of their degradative potential when unleashed through cellular disruption, many researchers are becoming aware of the problems which accompany unheeded proteolysis. There is perhaps no single class of enzymes that has more influence, practically speaking, on the outcome of protein research than do the proteases, for inattention to their deleterious effects can lead to the investigation of biologically irrelevant proteins.

This dissertation supports the hypothesis that multiple proteases exist in *Neurospora crassa*. The implications of such an existence warrent further investigation, both in *Neurospora* and other organisms. Efforts should involve the isolation and characterization of the observed proteolytic activities, localization of these activities within the organism, and development and isolation of mutants expressing altered protease and/or peptidase activities. In this manner the

number and character of the presumed multiple intracellular proteases can be determined, and an investigation into the cellular functions of these enzymes can begin.

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