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Limited Proteolysis in Microorganisms

THE AROM ENZYME CONJUGATE OF NEUROSPORA: MULTIPLE PROTEASES AND SUBUNIT ARTIFACTS[1]

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ABSTRACT

The arom enzyme conjugate of Neurospora crassa is a multifunctional protein consisting of five enzymes which catalyze a sequence of reactions leading to the biosynthesis of the aromatic amino acids. Previously, it was believed that this multienzyme system consisted of a set of physically associated but noncovalently linked enzymes. We now have good evidence that all five activities reside on a single polypeptide chain, and electron micrographs confirm that the enzyme conjugate is a dimer of this multifunctional polypeptide. The enzyme conjugate is very susceptible to proteolytic attack. However, a number of discrete proteolytic clips can occur before the system loses either its overall native structure or any one of its five activities. The uncontrolled action of a multiplicity of resident proteases in N. crassa during the extraction and purification of the conjugate led us to the false conclusion that this enzyme system consisted of noncovalently linked enzymes. Which of the many proteases that appear to be present in N. crassa are primarily involved in forming the artifactual subunits remains to be determined.

INDEX TERMS

Protease inhibitors; multifunctional protein; aromatic amino acids

The arom enzyme conjugate of Neurospora crassa consists of five covalently linked enzymes that catalyze sequential steps in the biosynthesis of the aromatic amino acids (Fig. 1). We have termed such multifunctional proteins multienzyme conjugates (3), thereby distinguishing them from multienzyme complexes or enzyme aggregates which consist of physically associated but noncovalently linked enzymes. The arom conjugate is made up of two identical polypeptide chains, each having a molecular weight of approximately 150,000 daltons (4, 5). From genetic analyses (9) the order of the enzymes on the polypeptide, starting from the amino terminus, appears to be enzyme 1 in the biosynthetic sequence followed by enzymes 5, 4, 2, and 3 (Fig. 1). The dimeric structure of this pentafunctional conjugate has been confirmed by Lumsden and Coggins (8), and, as shown in Fig. 2, we have obtained further confirmation by electron microscopy (Allison and Gaertner, in preparation).

F-1

F-2

Initially we (2) and others (6) believed that the arom conjugate (previously referred to as the arom aggregate or complex) existed as a system of noncovalently linked enzymes. This belief was supported by the fact that after the protein was purified, subunits were found with molecular weights much lower than 150,000 daltons (2, 6). Also, active fragments with similarly low molecular weights have been observed (1, 6). More recently we have shown that these "subunits" were artifacts produced by the action of resident proteases in N. crassa during the extraction and purification of the conjugate (4).

Table 1 shows two methods of purification. The first is slow, taking as long as 10 days to complete. The second can be accomplished in 3 days. Both procedures lead to a homogeneous preparation that exhibits a single band on native disc gel

T-1

electrophoretograms. However, under denaturing conditions with sodium dodecyl sulfate, the two procedures show different results. When the rapid procedure is used, the purified enzyme exhibits an electrophoretic band with a molecular weight of ~150,000 daltons; but when the slow method is used, a series of four faster moving bands with molecular weights totaling ~300,000 are obtained (2, 4, 5). In addition, if the rapid purification procedure is done with a protease inhibitor (phenylmethane-sulfonyl fluoride, PMSF) or if other measures are taken to avoid proteolysis, only the high-molecular-weight (150,000 daltons) band is observed (5). Although PMSF does allow for the purification of the intact conjugate (5, 8), it is not entirely satisfactory as an agent to prevent proteolysis since it inactivates two of the enzymes (numbers 4 and 5) in the arom enzyme system (Cole and Gaertner, in preparation).

Initial experiments on the proteolytic activity in N. crassa revealed a single major PMSF-sensitive peak of protease activity on either DEAE-cellulose or gel filtration chromatography (7). In addition, one of the five protease activities described by Siepen et al. (10) appeared to match the properties of this activity (7). As shown in Figs. 3 and 4, we have since learned that there are many more than five proteolytic activities in N. crassa (Spady and Gaertner, in preparation) and that it is therefore likely that many more than one protease is involved in the formation of the artifactual subunits. F-3 F-4

Figs. 3 and 4 also show several other interesting facts concerning the protease activity in N. crassa. First, there appears to be less proteolytic activity in early log-phase cultures of N. crassa than in late log- or stationary-phase cultures. However, it is also obvious that much of the proteolytic activity is masked, since,

after the samples have been stored, acidic, neutral, and alkaline protease activities appear throughout the DEAE-cellulose columns. Nonetheless, in isolating the arom enzyme conjugate it has proven advantageous to harvest the cells in the early log phase of growth. Because we (i) use 0.1 M potassium phosphate (pH 7.0) in our purification of the conjugate, (ii) use the 40–50% ammonium sulfate fraction, (iii) isolate the conjugate from DEAE-cellulose fractions 75 through 100, and (iv) use the early log-phase culture, we avoid much of the potential protease activity. It is also fortunate that the ammonium sulfate fraction that is optimum for isolating the conjugate is also the lowest in neutral proteolytic activity under these conditions. Additional experiments on the proteolytic activities in N. crassa (Spady and Gaertner, in preparation) have shown that most, if not all, can be inactivated by specific heat-stable peptide inhibitors from N. crassa. It is likely that these inhibitors account for the masking of the protease activities and that the inhibitors are simply more labile to storage than are the proteases.

In addition, we have found that only some of the neutral and alkaline proteases are inhibited by PMSF, but all of the acidic activities are inactivated by this reagent. Also, some of the proteases are inactivated by the commercial inhibitors benzamidine and Trasylol. However, most are not affected by these reagents, and several of them instead are remarkably activated. Moreover, based on a combination of criteria, including chromatographic location, pH optima, growth phase, cryptic behavior, and response to natural and commercial inhibitor preparations, we have accounted for at least 25 distinct proteases in N. crassa, and from chromatographic position alone our data suggest that there may be well over 100 distinct peptidases in this organism. If

these observations are substantiated, the implications with respect to the regulatory roles that such a multiplicity of specifically inhibitable proteases play in cellular metabolism will clearly be of major importance and no doubt will be applicable to other eukaryotic organisms.

Limited proteolysis of the arom conjugate by inadvertent events (2, 4) and under controlled conditions (Vitto and Gaertner, in preparation) has revealed several facts concerning the structure and function of this system. First, it is clear that a number of proteolytic clips can occur before any of the enzymes in the conjugate are inactivated (4). Also, we have shown that the enzymes remain physically associated so that the system eventually takes on the appearance of a complex of noncovalently linked enzymes rather than a multifunctional protein. However, after an indeterminant number of proteolytic clips have occurred, the system begins to lose activity and the native structure begins to disintegrate into fragments (4, 7). Some of these fragments apparently can retain one or more of the conjugate's activities (1, 6).

The initial limited proteolytic events seem to result in very discrete polypeptide fragments (2, 4), and the artificial subunits that are formed initially seem to be very similar, regardless of the endopeptidase used (Vitto and Gaertner, in preparation). These results are consistent with the concept of separate domains or tightly folded regions of the polypeptide that are joined by exposed protease-sensitive regions. Presumably each domain is responsible for catalyzing one of the five enzyme activities in the conjugate, but whether or not this is in fact the case remains to be determined.

TEXT FOOTNOTES

[1] Research supported in part by Grant No. PCM76-80227 from the National Science Foundation and by the Division of Biological and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

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TABLE 1

Purification of the arom enzyme conjugate*

Purification steps	Total volume (ml)	Total protein (A_{280})	Total DHS reductase† (units)	Recovery (%)	Specific activity (units/A_{280})
Method I					
1. Crude extract	8,800	~200,000	~110	100	~0.0006
2. Protamine and $(NH_4)_2SO_4$	171	17,800	110	~100	0.0062
3. Sephadex G-25	350	—	—	—	—
4. DEAE-cellulose (4 X 90 cm)	650	1,560	75.4	69	0.048
5. Concentration by $(NH_4)_2SO_4$	20	1,240	77.5	70	0.062
6. Sephadex G-75	45	378	32.6	30	0.086
7. DEAE-cellulose (2 X 80 cm)	290	267	25.7	28	0.096
8. Concentration by $(NH_4)_2SO_4$	10	190	21.0	19	0.111
9. Sephadex G-200	38	123	17.1	15	0.139
10. Preparative electrophoresis	35	3.2	8.5	8	2.7

(Table 1 continued)

(Table 1 continued)

Method II					
1. Crude extract	2,200	~50,000	~34	100	~0.0007
2. Protamine and $(NH_4)_2SO_4$	55	4,950	34	~100	0.007
3. Sephadex G-25	175	–	–	–	–
4. DEAE-cellulose (2 X 80 cm)	170	388	22	63	0.057
5. Phosphocellulose	105	74	20	57	0.270
6. Cibacron blue Sepharose	15	9	21	60	2.3

*Data from ref. 2.

†Dehydroshikimate (DHS) reductase is one of the five activities of the enzyme system.

FIGURE LEGENDS

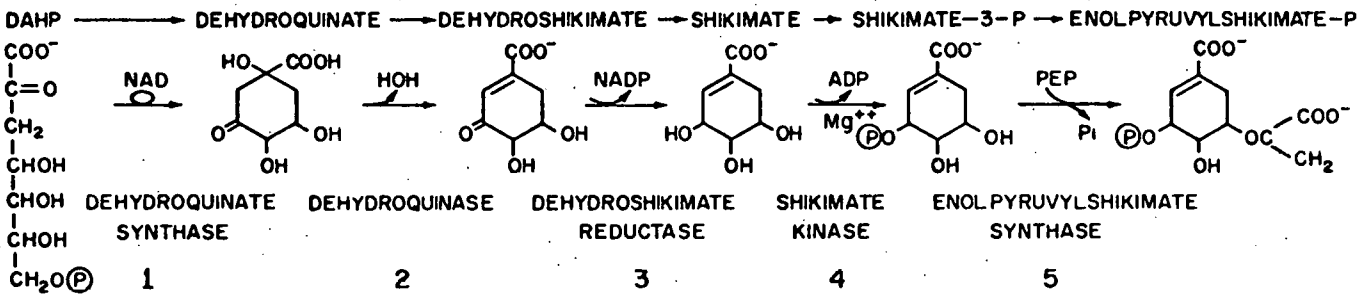
Fig. 1. The polyaromatic pathway and the arom enzyme conjugate.

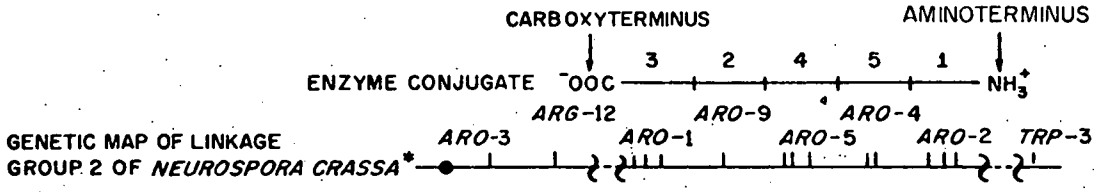
Fig. 2. Electron micrographs of the arom conjugate. Areas were cut from a 10,000-Å square field showing the dimeric structure of the system. Below each micrograph is an artist's representation of the dimer. For purposes of electron microscopy the enzyme, in 0.1 M potassium phosphate, was diluted 1:50 with water, placed on carbon-coated grids, and stained with uranyl acetate.

Fig. 3. 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 described in ref. 2. The chromatograms (2 X 80 cm) were assayed with azocoll at pH 9 (···), pH 7 (---), and pH 5 (——). The starting material was 150 g lyophilized mycelium, and the percentage saturation with ammonium sulfate was: A–D, 0–40%; E–H, 40–50%; and I–L, 50–80%. D, H, and L show proteolytic activity in the three ammonium sulfate fractions immediately after chromatography. C, G, and K show the activity after a 1-ml aliquot was frozen and thawed approximately 10 times over a 6-month period. B, F, and J show the activity after the sample was stored in 20-ml plastic vials for 6 months at -10°C. A, E, and I show the proteolytic activity after 10 month's storage under the same conditions.

Fig. 4. Effects of freeze/thaw and storage at -10°C on the proteolytic activity in fractions from early log-phase N. crassa. See Fig. 3 for further description.

34522

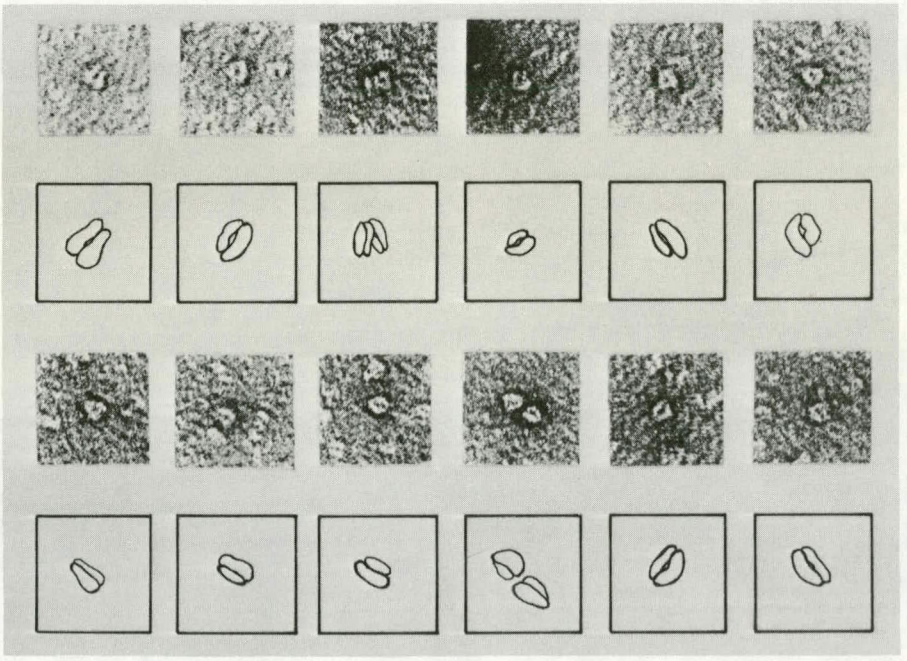




*DATA FROM RINES, CASE AND GILES, GENETICS, 61 (1969) 789.

Fig 1

34524



1,000 Å

fig 2

34197

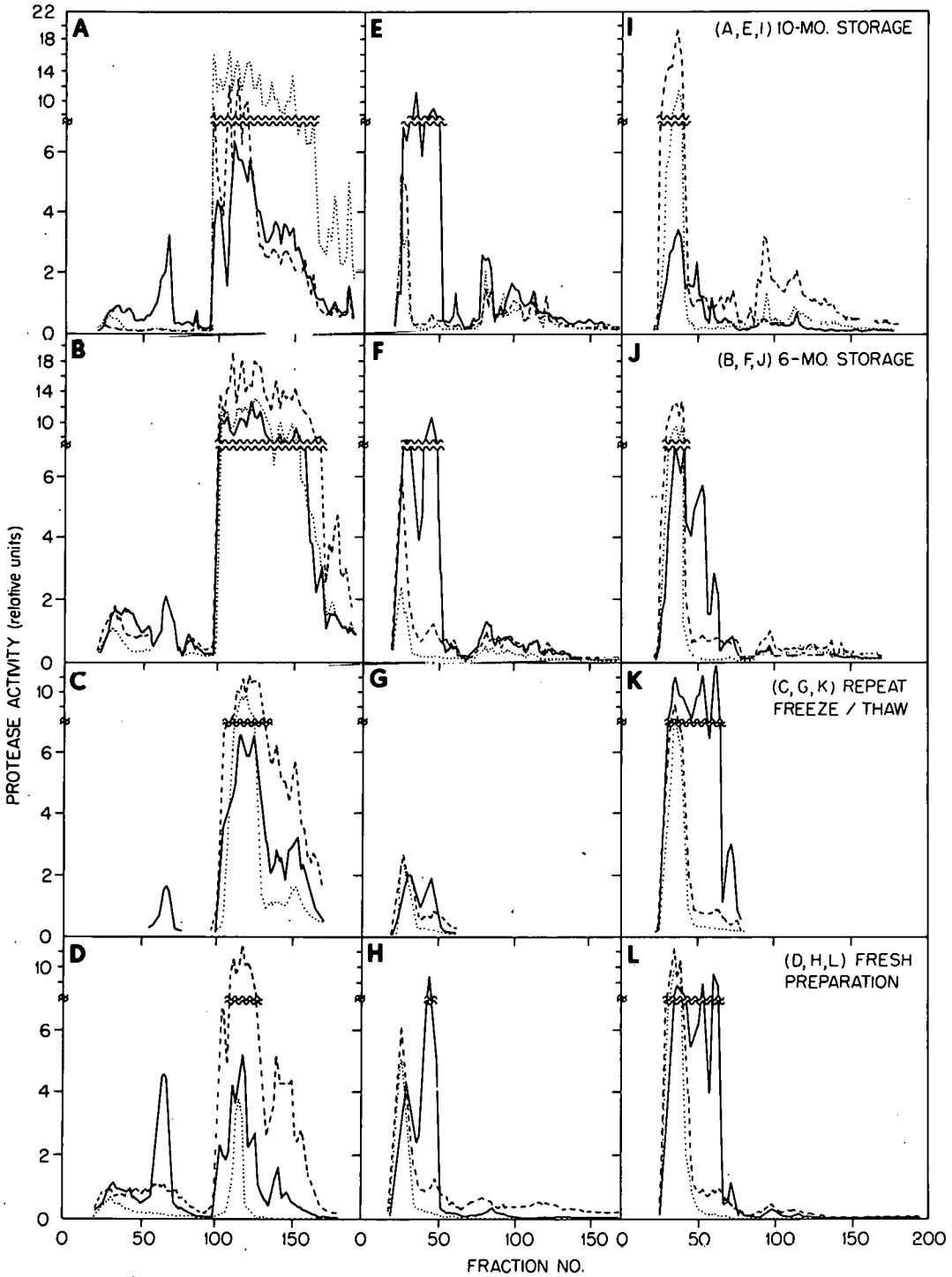

A
E
I
(A,E,I) 10-MO. STORAGE
B
F
J
(B, F,J) 6-MO. STORAGE
C
G
K
(C, G, K) REPEAT FREEZE / THAW
D
H
L
(D, H, L) FRESH PREPARATION
PROTEASE ACTIVITY (relative units)
FRACTION NO.
0
2
4
6
10
14
18
22
50
100
150
200

Fig. 3

34198

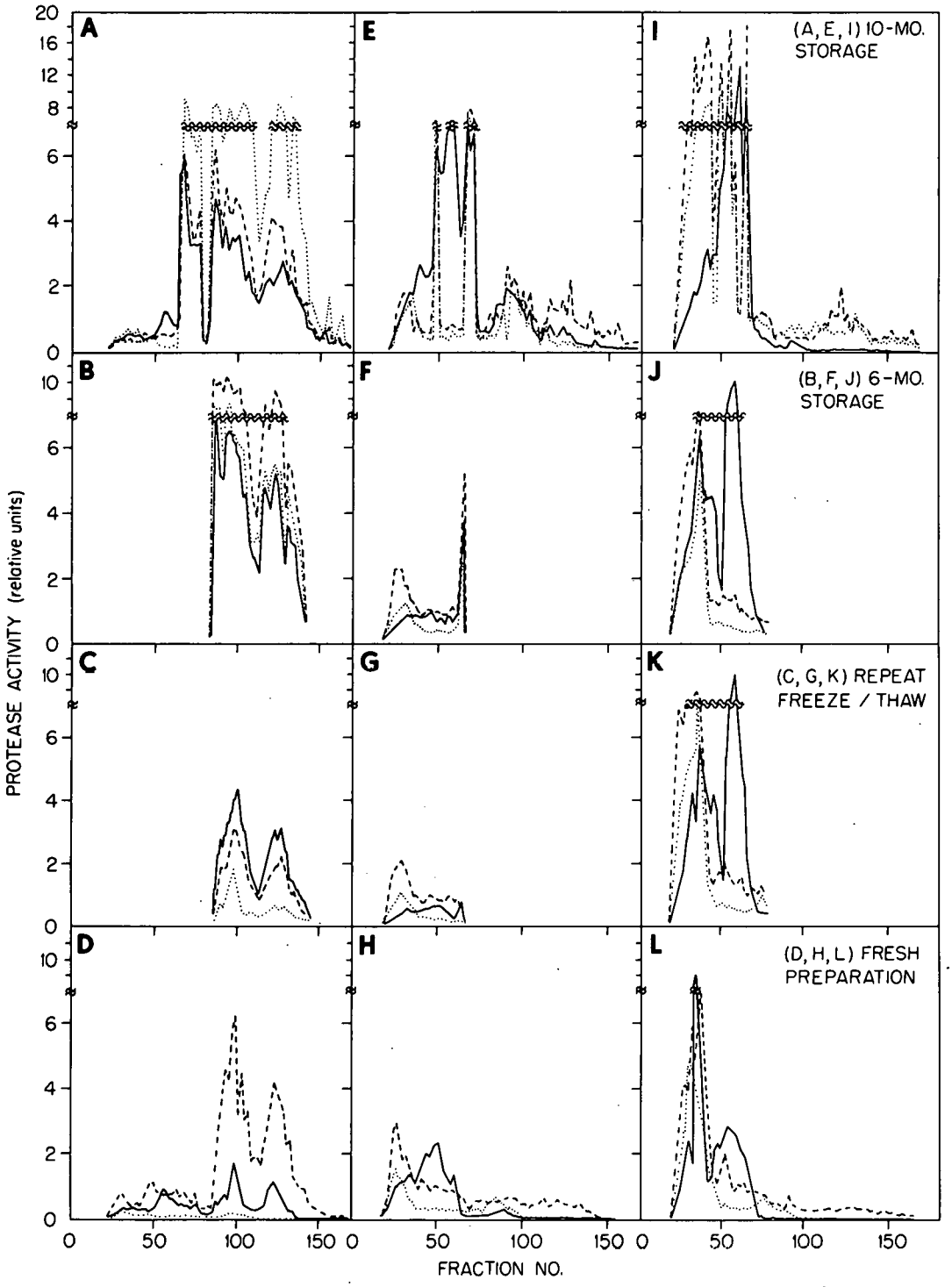

A
E
I
(A, E, I) 10-MO. STORAGE
B
F
J
(B, F, J) 6-MO. STORAGE
C
G
K
(C, G, K) REPEAT FREEZE / THAW
D
H
L
(D, H, L) FRESH PREPARATION
PROTEASE ACTIVITY (relative units)
FRACTION NO.
0
50
100
150
2
4
6
8
10
12
16
20

Fig. 4