PROTEINASE MUTANTS OF *SACCHAROMYCES CEREVISIAE*[1]

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ABSTRACT

Fifty-nine mutants with reduced ability to cleave the chymotrypsin substrate N-acetyl-DL-phenylalanine β-naphthyl ester have been isolated in *S. cerevisiae*. All have reduced levels of one or more of the three well-characterized proteinases in yeast. All have reduced levels of proteinase C (carboxypeptidase Y). These mutations define 16 complementation groups.

THREE proteinases that have been characterized in yeast are proteinases A and B (HATA, HAYASHI and DOI 1967a,b; LENNEY and DALBEC 1967; SAHEKI and HOLZER 1974) and proteinase C, also called carboxypeptidase Y (DOI, HAYASHI, and HATA 1967; HATA, HAYASHI, and DOI 1967b; HAYASHI, AIBARA, and HATA 1970; HAYASHI, MOORE and STEIN 1973; KUHN, WALSH and NEURATH 1974). Macromolecular inhibitors for each of these proteinases are found intracellularly (BETZ, HINZE and HOLZER 1974; MATERN, HOFFMAN and HOLZER 1974; SAHEKI, MATSUDA and HOLZER 1974). The proteinases appear to be localized in vesicles (CABIB, ULANE and BOWERS 1973; HASILIK, MÜLLER and HOLZER 1974; LENNY *et al.* 1974; MATERN, BETZ and HOLZER 1974), whereas the inhibitors for proteinase B (HASILIK, MÜLLER and HOLZER 1974; LENNEY *et al.* 1974; MATERN, BETZ and HOLZER 1974), proteinase A (LENNEY *et al.* 1974; MATERN, BETZ and HOLZER 1974) and proteinase C (MATERN, BETZ and HOLZER 1974) are localized in the cytoplasm. Based on *in vitro* observations, proteinases A and B have been implicated in inactivation of tryptophan synthetase (KATSUNAMA *et al.* 1972; SAHEKI and HOLZER 1974; SAHEKI and HOLZER 1975), activation of chitin synthetase (CABIB and FARKAS 1971; CABIB and ULANE 1973; CABIB, ULANE and BOWERS 1974; HASILIK and HOLZER 1973), and proteinase B has been postulated to participate in septation in yeast (CABIB, ULANE and BOWERS 1974).

Protein degradation and turnover has been shown to occur when cells undergo meiosis and sporulation (ESPOSITO *et al.* 1969; HOPPER *et al.* 1974) and the levels of the three proteinases rise when cells sporulate (BETZ and WEISER 1976; KLAR and HALVORSON 1975; CHEN and MILLER 1968).

The role of these three proteinases in the physiology of the cell and in the transition between cell states (mitosis and meiosis) should be clarified if mutants

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altered in these activities can be isolated. In this paper, I report isolation of mutants deficient in one or more of these proteinases.

MATERIALS AND METHODS

Yeast strains: Haploid yeast strains used in this work were a3–41 (**a** *ade3–41 leu1–1*) and M16–14C (**a** *ser1–171 leu1–1*). All mutants were derived from these strains. The outcross parent was C1001–146–1Ad+16 (*α ade2–1 trp5–2 lys2–1 ura3 cyh2 can1–11*).

Media: YEPD, SC, omission media and sporulation medium are as previously described (JONES and LAM 1973).

Growth conditions and preparation of extracts: Cells were grown in YEPD for 48 hours at 30°, were harvested by centrifugation, washed once with distilled water and resuspended in 0.1 M Tris HCl buffer (pH 7.6). Two ml of buffer were added for each gram of cells. The cells were broken (3 min) with 0.45 mm glass beads (equal volumes cell suspension and glass beads) in a homogenizer (Braun-Melsungen, West Germany). The suspension was centrifuged 30 min at 35,000 × *g* in a Sorvall RC5 centrifuge. One portion of the supernatant solution was brought to pH 5 with 3 N acetic acid for assay of proteinases A and C (the latter after incubation at room temperature for 8 hrs). A second portion was brought to 0.26% in sodium dodecyl sulfate (SDS) by addition of an appropriate amount of 20% SDS, pH 7.6. This preparation was immediately used for assay of proteinase C. After 6 hr incubation at room temperature, this SDS treated extract was used for assay of proteinases B and C.

Proteinase activity assays: Proteinase A activity was measured according to LENNEY *et al.* (1974), using hemoglobin as substrate. The perchloric acid-soluble products released by proteinase A action were measured according to LOWRY *et al.* (1951). One unit is defined as 1 μg of tyrosine-containing peptides released into the supernatant per min per mg of protein at 37°. Acid denatured hemoglobin was prepared by dissolving 2.5 g hemoglobin (Sigma Chemical Co.) in 100 ml distilled water. This solution was dialyzed against three changes of three liters of water in the cold. The pH was brought to 1.8 with N HCl. After 1 hr incubation with stirring at 35° the pH was brought to 3.2 with N NaOH. The volume was then adjusted to 125 ml. Proteinase B was assayed according to JUNI and HEYM (1968) with the following modifications: 0.05 ml extract was added to a tube containing 0.35 ml of 0.1 M Tris HCl pH 7.6, 0.1 ml of 1% Triton X-100 and 20 mg azocoll (Calbiochem) at 37°. After 20 min, 3.5 ml of ice water were added. After centrifugation, the absorbance of the supernatant was determined at 520 nm. If the extract is pretreated with SDS as described above and the tubes are shaken, the kinetics are linear with time and protein concentration. One unit is defined as a change of one absorbance unit at 520 per min per mg of protein. Proteinase C was assayed according to AIBARA, HAYASHI, and HATA (1971) with the following modifications: 0.1 ml of 6 mM N-benzoyl-L-tyrosine-p-nitroanilide (BTPNA) (Sigma Chemical Co.) in dimethylformamide (DMF) was added to a tube containing 0.45 ml 0.1 M Tris HCl pH 7.6 and 0.05 ml extract at 37°. After 30 min, 1 ml of 1 mM $HgCl_2$ was added, followed by 0.5 ml water and 0.2 ml of 20% SDS pH 7.6. After vortexing, the tubes were incubated at 70° until the solutions became clear due to solubilization of the protein. The absorbance at 410 nm was determined. One unit of activity corresponds to 1 μmole p-nitroaniline produced per min per mg, assuming a molar absorbance of 8800. Corrections for absorbance due to substrate and protein were made.

Gel electrophoresis: Proteins were separated by slab electrophoresis on acrylamide gels with a continuous buffer system in the apparatus described by STUDIER (1973). The gels have an acrylamide:bis ratio of 30:0.8, and polymerization was initiated by N,N,N′,N′-tetraethylmethylenediamine, added to a final concentration of 0.05%, and ammonium persulfate, added to a final concentration of 0.1% (Eastman Kodak). The gel contained 0.05 M Tris HCl pH 7.6, 4.5% acrylamide. The running buffer was 0.05 M Tris HCl pH 7.6. Extracts were brought to pH 5 with 3 N acetic acid, were incubated 2 hrs at 37° and then overnight at 4°. Aliquots of activated extract were mixed with an equal volume of 0.06 M Tris HCl pH 7.6, containing 0.02 mg/ml bromphenol blue and 62.5% sucrose w/v. Twenty μl of the mixture were layered in each well. Electrophoresis was at 100V for 210 min.

Gel stain for esterolytic activity: 60 mg of N-acetyl-DL-phenylalanine β-naphthyl ester (APE) was dissolved in 30 ml DMF. This was added to 120 ml Tris HCl buffer pH 7.3 such that the final buffer concentration was 0.05 M. After 15 min incubation at room temperature, 150 mg of Chroma-Gesellschaft Fast Black K Salt (Roboz Surgical Instrument Co., Inc.), dissolved in a minimal volume of water, was added to the substrate solution and was mixed by swirling. After 30 min further incubation, the staining solution was decanted and the gels were repeatedly washed with distilled water.

Mutagenesis: 0.3 ml ethylmethane sulfonate (Eastman Organic Chemicals) was added to cells suspended in 10 ml of 0.05 M phosphate buffer pH 8.0. After 60 or 90 min shaking at 30°, an aliquot of the suspension was diluted into 6% sodium thiosulfate. Further dilutions were made in $Na_2S_2O_3$. Cells were plated onto YEPD to a colony density of 100–200 per Petri dish. After growth at 30° for 2 days, the plates were transferred to 36° for 30 hr. Mutants with reduced ability to cleave APE (Schwarz/Mann or Bachem) were isolated. Enzymatic activity resulting in cleavage of APE was visualized according to the following procedure. To 3 ml of molten 0.6% agar at 50° was added 2 ml of DMF containing 6 mg of APE. After mixing, the agar was poured over colonies on the plate. After the agar solidified (10 min), 3.5–4.0 ml of 0.1 M Tris HCl pH 7.3 containing 5 mg/ml Fast Garnet GBC (Sigma Chemical Co. or Roboz Surgical Instrument Co., Inc.) was gently poured over the surface of the agar. Within a few minutes the colonies turned red; mutant colonies turned red much more slowly. The diazonium salt solution was poured off as soon as the colonies started to turn red. Mutant colonies were stabbed with a sterile toothpick and were streaked on YEPD. After a second reisolation, colonies were retested for cleavage ability using the above procedure.

Genetic analyses: Procedures for sporulation and tetrad dissection have been previously described by HAWTHORNE and MORTIMER (1960). For complementation analyses, diploids were isolated by prototrophic selection. Diploid clones were streaked on YEPD, as were the input parents. After growth at 30° for 2 days, the clones were replica plated to YEPD (LEDERBERG and LEDERBERG 1952) and to omission media for scoring of other markers. After 2 days at 30°, APE cleavage ability was determined using the method described for mutant isolation. However, the APE concentration was reduced to ⅓ of that given above.

RESULTS

Complementation groups defined by 59 mutants deficient in ability to cleave APE:

Two of the proteinases found in yeast, namely proteinases B and C, have esterolytic activity toward acetyltyrosine ethyl ester (HASILIK, MÜLLER and HOLZER 1974). N-Acetyl-DL-phenylalanine β-naphthyl ester is a known substrate for chymotrypsin (HAAS, ELKANA and KULKA 1971). We anticipated that one or both of the yeast proteinases might cleave APE. This was later shown to be the case for proteinase C (WOLF and FINK 1975). We thus sought mutants with a reduced ability to cleave APE. From about 3×10^4 colonies, a total of 59 mutants with reduced rates of cleaving APE were isolated in two strains. Five randomly chosen mutants were crossed to C1001–146–1 Ad+16 in order to isolate the mutants in the opposite mating type. The mutations segregated as single mutations, although a modifier of the mutations was also seen to segregate. The five strains isolated from the outcrosses, each bearing one of the five mutations, were crossed to the original wild-type parents (M16–14C and a3–41) and to the 59 mutants derived from them. The resulting diploids, isolated by prototrophic selection, were tested for APE cleavage ability to determine whether or not the five mutations complemented one another or the other 54 mutations. The domi-

TABLE 1

Distribution of mutations with reduced ability to cleave APE among 16 complementation groups

	prc1	*pep1*	*pep2*	*pep3*	*pep4*	*pep5*	*pep6*	*pep7*	*pep8*	*pep9*	*pep10*	*pep11*	*pep12*	*pep13*	*pep14*	*pep15*	Total
3–41	10	4	0	4	1	2	1	0	0	1	1	0	0	0	0	0	24
M16–14C	13	4	3	1	1	1	3	3	1	0	0	1	1	1	1	1	35
Total	23	8	3	5	2	3	4	3	1	1	1	1	1	1	1	1	59

nance relationships were also determined. All five tester mutations were recessive. The five mutations fell into two complementation groups (three in one, two in the other), giving unequivocal and internally consistent results. Unequivocal and internally consistent results were also obtained in crosses to the other 54 mutant strains. Twenty of the 54 mutants fell into the first complementation group bringing the total to 23; six additional mutants fell into the second complementation group for a total of 8. These two complementation groups account for more than half of all mutants isolated (31/59) (see Table 1). One can also infer from the above results that the 28 mutations which complemented the mutations in the five tester strains are recessive, for the fact of complementation necessitates this conclusion. Hence these data indicate that all 59 mutants isolated are recessive.

A second group of five mutants was selected from among the 28 mutants which complemented the first five testers, and the cycle of outcrossing and testing was repeated. This procedure was repeated until all mutants had been assigned to complementation groups. It is important to note, however, that not all mutants have been crossed to one another. The results of these complementation analyses define 16 complementation groups. The distribution of mutations within the groups is given in Table 1 for each of the two parent strains. The *prc1–1* mutation (WOLF and FINK 1975) fails to complement the 23 mutations in our first group, but complements all other mutations. The additional 15 complementation groups have been designated *pep1* to *pep15*, for all lead to reduced levels of one or more peptidases, as will be shown below.

Proteinase levels in prc *or* pep *mutants:*

We have determined proteinase levels in one representative of each of the 16 complementation groups defined above (Table 2). The procedures that we have used to activate the extracts differ significantly from published procedures (BETZ and WEISER 1976; SAHEKI and HOLZER 1975). In previous work, the extracts were activated by incubation at pH 5 for periods ranging from 18 to 24 hrs. Our activation procedure involves incubation in 0.26% SDS at pH 6.7 for 6 hrs. For proteinase B, SDS activation is essentially complete within 6 hrs, and the activity remains essentially constant for 2 additional hours at least (data not shown). For extracts activated in this fashion, the assay is linear with time for at least 30 min. SDS treated extracts assayed for proteinase C after little time in SDS (SDS_0) show activities comparable to those for acid activated extracts. After 6 hrs of SDS treatment there is a slight activation (20–25%) of proteinase C for wild-type strains. (See strains a3–41 and M16–14C in Table 2). The data in Table 2 show that the proteinase C activities demonstrable in our parent strains (or mutants in some cases) are much higher after 6 hrs of SDS activation than they are after 8 hrs incubation at pH 5.

Specific activities for the two parent strains, and for the mutants derived from them, are given in Table 2. The mutant strains are isogenic with their parent strains. All mutants, without exception, have reduced levels of proteinase C. This observation agrees with WOLF and FINK's observation that proteinase C is the

TABLE 2

*Specific activities of proteinases A, B and C in crude extracts of the parent strains a3–41 and M16–14C and mutant strains derived from them**

Strain	proteinase A U/mg	proteinase B U/mg	proteinase C mU/mg		
			SDS_0	SDS_6	$pH5_8$
a3–41†	4.45	0.0219	1.87	2.56	1.37
prc1–2	4.15(93)‡	0.0188(86)‡	0	0.03(1)‡	0
pep1–1	3.75(85)	0.0327(149)	0	0.96(38)	0
pep3–1	2.32(52)	0	0	0.05(2)	0
pep4–1	0.13(3.0)	0	0.04	0.04(2)	0
pep5–1	1.84(42)	0.00015(0.7)	0.17	0.18(7)	0.18
pep6–1	4.00(90)	0	0	0.11(4)	0
pep9–1	3.52(79)	0.0196(89)	0.70	1.93(75)	0.54
pep10–1	4.02(90)	0.0180(82)	0.35	1.38(54)	0.25
M16–14C†	3.81	0.0203	2.12	2.56	1.89
pep2–1	2.84(75)	0.0060(30)	0.31	0.71(28)	0.25
pep7–1	1.50(34)	0	0	0	0
pep8–1	2.19(58)	0	0.40	0.55(22)	0.34
pep11–1	2.34(62)	0.0080(39)	0	0.02(1)	0
pep12–1	1.40(37)	0	0	0.02(1)	0.06
pep13–1	3.87(102)	0.0011(5)	0.38	0.69(27)	0.34
pep14–1	1.60(42)	0	0.08	0.14(5)	0.01
pep15–1	2.17(57)	0.0045(22)	0.48	0.52(20)	0.27

* Proteinase A activity was measured against hemoglobin, proteinase B against azocoll, proteinase C against BTPNA. Proteinase C activity was determined after incubation of the extract in 0.26% SDS at room temperature for 0 or 6 hrs, or after acidification to pH5 and incubation at room temperature for 8 hrs.

† Mutants listed under a3–41 were derived in that strain, under M16–14C in that strain.

‡ Numbers in parenthesis are % of parent specific activity. For proteinase C, % of parent specific activity after 6 hrs SDS treatment.

only protease capable of cleaving APE (WOLF and FINK 1975), and these mutants were specifically isolated as having reduced ability to cleave APE. The deficiency is essentially absolute for some mutant strains (*prc1–2*, *pep3–1*, *pep4–1*, *pep6–1*, *pep7–1*, *pep11–1*, *pep12–1*). For the other strains only a partial deficiency is evident. Mutants with absolute deficiencies for proteinase C can be subdivided into those which lack only proteinase C (*prc1–2*), those lacking proteinases B and C (*pep3–1*, *pep6–1*, *pep7–1*, *pep12–1* and *pep14–1*, and those lacking all three proteinases (*pep4–1*).

prc1–2 has the enzymatic phenotype expected for a strain bearing a mutation allelic to *prc1–1* (WOLF and FINK 1975), for it has essentially normal levels of proteinases A and B, but little or no activity for proteinase C.

Strains bearing mutations in five different complementation groups (*pep3–1*, *pep6–1*, *pep7–1*, *pep12–1*, and *pep14–1*) have little or no activity for proteinase C and no demonstrable activity for proteinase B. Mutants with this enzymatic phenotype (with the exception of *pep6–1*) have levels of proteinase A roughly 40–50% of their parental strain levels. This may reflect the fact that proteinase B is capable of inactivating the inhibitors of proteinase A (SAHEKI, MATSUDA, and

HOLZER 1974). That these are not all mutations in the same complementation group that might have been misassigned is evidenced by the observation that all strains bearing alleles in *pep3* are unable to utilize glycerol as a carbon source, but can use acetate. Mutations in the other four complementation groups do not interfere with glycerol utilization. The mutations *pep12–1* and *pep14–1* each show linkage to their centromeres; mutations in the other three do not (unpublished observations). Within these five complementation groups, then, there are at least three distinct classes.

One of the mutants, *pep4–1*, appears to lack proteinases A, B, and C. The parent strain of *pep4–1*, *a3–41*, requires leucine, as does M16–14C. This need can be satisfied by N-Cbz-leucyl-leucine for the two parent strains and for all *prc* or *pep* mutants derived from them, with the exception of *pep4–1*. Growth of *pep4–2* was not tested. Possibly *pep4–1* lacks an additional carboxypeptidase present in the two parents and other mutants.

Two of the mutants (*pep5–1* and *pep8–1*) have very low levels of proteinase B, the low proteinase A levels typical of mutants lacking B activity, and low levels of proteinase C. We have reason to doubt that these mutants truly lack B activity, for B activity can be detected if the activation period is prolonged.

The remaining seven mutants do not fall into clean phenotypic groupings, for they may have high B or low C levels (*pep1–1*), intermediate to low levels of B or C (*pep2–1*, *11–1* or *13–1*), low but SDS activatable levels of C (*pep9–1*, *pep10–1*), or intermediate levels of all three proteinases (*pep15–1*).

It is clear that a substantial proportion of the mutants described are pleiotropic, in that more than one of the proteinases is totally absent. (B^-C^- or $A^-B^-C^-$ mutants). Segregation of the mutant alleles has been monitored by assessing the ability of clones to cleave APE. This means that we are following segregation of the deficiency for proteinase C. We are currently performing the analyses to determine whether or not the multiple proteinase deficiencies segregate together, but we are unable to report these analyses at this time.

All of the mutants described above lack, or are deficient in, proteinase C activity as measured in crude extracts. Some appear to lack proteinase B as well, and one lacks proteinases A, B, and C. It is of considerable importance to know whether these mutants truly lack the enzyme(s), or whether the deficiencies observed reflect a failure to activate the enzymes by destruction of the proteinase inhibitors. This determination can and has been made for proteinase C, but not for proteinases A and B. As reported by WOLF and FINK (1975), one can detect proteinase C, whether free or bound to its inhibitor, after electrophoresis of cell-free extracts on acrylamide gels with subsequent staining for APE cleavage activity. After electrophoresis of wild-type extracts (activated at pH 5), one finds three bands of APE cleavage activity, a slow narrow band (1) which is not inhibited by phenylmethylsulfonyl fluoride (PMSF) and two bands of intermediate mobility (2 and 3), each of which is inhibited by PMSF. Each of the PMSF sensitive bands cleaves the proteinase C substrate BTPNA and N-Cbz-leu-leu. The fastest band (3) corresponds to proteinase C, the middle band (2) to proteinase C complexed to its inhibitor. If a mutation eliminates bands 2 and

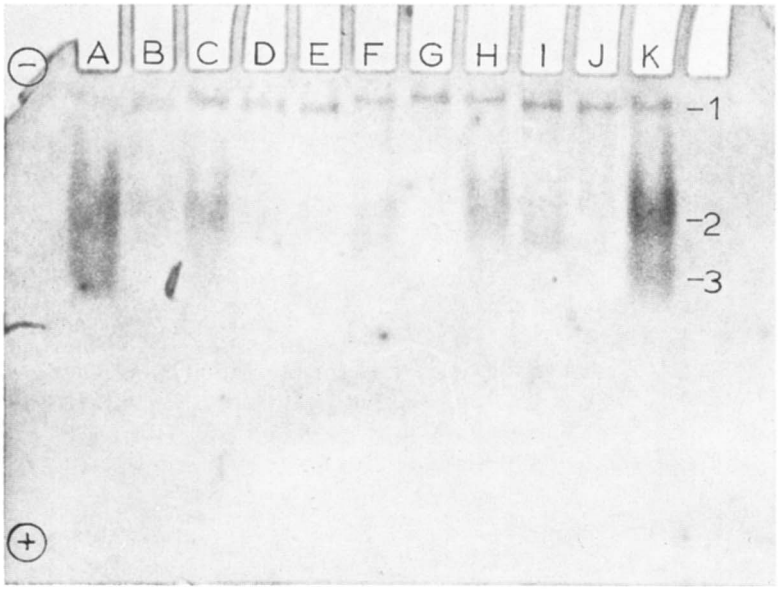

FIGURE 1.—Slab electrophoresis of activated extracts of a3–41 and mutants derived from it with subsequent staining for APE cleavage activity. Bands 1, 2 and 3 correspond to an unknown esterase, proteinase C complexed with proteinase C inhibitor, and free proteinase C, respectively. Extracts present in the well are A and K, *a3–41*; B, *pep6–1*; C, *pep5–1*; D, *pep4–1*; E, *pep5–2*; F, *pep1–1*; G, *prc1–2*; H, *pep9–1*; I, *pep10–1*; J, *pep3–1*.

3 of APE cleavage activity, the simplest interpretation is that proteinase C is truly absent, and not merely tied up in an inactive form in an enzyme inhibitor complex.

The gel patterns of APE cleavage activity present in cell-free extracts of *a3–41* and mutants derived from it can be seen in Figure 1. Cell-free extracts of *prc1–2* (well G), *pep3–1* (well J), *pep4–1* (well D) and *pep6–1* (well B) had essentially no proteinase C under any condition of activation (Table 2). These same mutants lack the bands 2 and 3 of APE cleavage activity (*pep6–1* has a faint band 2). Mutants *pep1–1* (well F), *pep5–1* (well C), *pep9–1* (well H) and *pep10–1* (well I) all showed proteinase C activity in cell-free extracts and have a band 2 corresponding to the enzyme-inhibitor complex. These gel patterns indicate that mutant strains which assay as proteinase C negative also lack the bands of APE cleavage activity associated with the proteinase C protein (whether free or complexed to inhibitor), and that the proteinase C negative phenotype does not reflect failure of activation. Similar results are obtained for mutant derivatives of M16–14C. Thus it seems likely that mutant strains which assay as proteinase C negative do indeed lack the enzyme.

DISCUSSION

The results presented in this paper show that mutations in any one of 16 different complementation groups can result in diminished specific activities for

proteinase C. Some of the effects on proteinase C may be direct effects, whereas others may be secondary effects. In view of the fact that inhibitors of the proteinases exist (BETZ, HINZE and HOLZER 1974; MATERN, HOFFMAN and HOLZER 1974; SAHEKI, MATSUDA and HOLZER 1974) and that these inhibitors can in turn be destroyed by one or more of the proteinases (SAHEKI, MATSUDA and HOLZER 1974), it is not entirely surprising that mutations in so many genes can lead to a diminution in proteinase C activity.

At first encounter one might expect the explanation of pleiotropic mutations to reside in the web of activations described for these enzymes. One might postulate that *pep4–1*, a mutation which results in absence of three, and possibly four proteinases, is really a mutation eliminating proteinase A activity (either by inactivation of proteinase A or by a change in structure or level of its inhibitors) and that the absence of proteinases B and C is a secondary consequence of the absence of A activity, and is attributable to the failure to activate (destroy the inhibitors of) proteinases B and C. This postulate cannot be refuted for the case of proteinase B. With respect to proteinase C, however, APE cleavage activity corresponding to proteinase C or to the proteinase C inhibitor complex was not detected in cell-free extracts of *pep4–1* subjected to electrophoresis on acrylamide gels, making it unlikely that proteinase C was present (but not activated) in the *pep4–1* mutant extracts. Similar arguments can be made for the B^-C^- mutants to eliminate the failure to activate proteinase C by proteinase B as the explanation for the C^- part of the B^-C^- phenotype.

A second obvious possible explanation of the pleiotropic mutations is that the genes for the proteinases are clustered, and that the B^-C^- and $A^-B^-C^-$ mutations are each classes of polar mutations. This hypothesis does not accord with the facts, for the two classes of pleiotropic mutations complement one another.

Among the viable hypotheses remaining are (1) the pleiotropic mutations are mutations in regulatory genes, (2) these pleiotropic mutations cause changes in the structure of the compartments containing these enzymes or (3) the pleiotropic mutations alter components of the system that places the enzymes in the compartments.

Many of the mutants appear to lack proteinase B, the enzyme which has been postulated to activate chitin synthetase to allow septation during vegetative growth (CABIB, ULANE and BOWERS 1974). Until we determine whether or not our B^-C^- and $A^-B^-C^-$ mutants actually lack the proteinase B enzyme, our results cannot be considered to refute this hypothesis.

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