ISOLATION OF YEAST MUTANTS LACKING PROTEINASE B ACTIVITY

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Received 23 May 1978

1. Introduction

The investigation of the function of proteolytic enzymes in cellular metabolism has gained wide interest in recent years as their great importance in cellular regulation in prokaryotes and eukaryotes has been realized [1]. However, until now we are, in most cases, far from understanding the specific roles of the different proteolytic enzymes found in an organism. In the yeast *Saccharomyces cerevisiae* seven intracellular proteolytic enzymes have been detected as yet (for a review see [2,3]). A crucial role in cellular regulation had been attributed to one of the endoproteinases, proteinase B. Cabib and coworkers had shown in vitro, that proteinase B is able to convert an inactive pro-form of chitin synthetase, an enzyme necessary for yeast budding and thus growth, to the active enzyme, and they hypothesized that proteinase B also regulates this reaction in vivo [4,5].

In vitro studies also suggested that proteinase B may be responsible for the catabolite inactivation of cytoplasmic malate dehydrogenase and fructose-1,6-bisphosphatase [6–8]. Proteinase B is also believed to play a central role in the differentiation process of sporulation [9,10]. Mutants lacking proteinase B should permit an evaluation of the hypothesized functions and uncover its role in cellular metabolism.

2. Materials and methods

The *Saccharomyces cerevisiae* strain we used in our experiments, was the temperature sensitive cell lysis mutant 233 (a, cly 4–1, ade 1, ade 2, ura 1, his 7, lys 2, tyr 1, gal 1) isolated by Hartwell and mentioned by Mortimer and Hawthorne [11]. Cells were grown in complete medium YPD containing 1% yeast extract (Difco), 2% peptone (Difco) and 2% glucose, supplemented with 135 mg/l adenine and 112 mg/l uracil. Solid medium was the growth medium supplemented with 2% agar (Difco). Liquid cultures were grown for 72 h at 23°C into stationary phase and aerated by shaking. For preparation of crude extracts, cells were harvested by centrifugation, washed with distilled water, suspended in 0.1 M potassium phosphate buffer, pH 7 (1:1, w/v, cells: buffer) and broken in a French pressure cell (Aminco). Mutagenesis was performed with ethyl methane sulfonate (Eastman Organic Chemicals) according to the procedure described by Fink [12]. Proteinases A and B were assayed as described by Saheki et al. [13]. Carboxypeptidase Y was measured according to Aibara et al. [14]. Proteinase B inhibitor activity was determined according to Betz et al. [15]. Malate dehydrogenase was measured as outlined in [16]. Purified proteinase B was a generous gift of Miss H. Hinze. The proteinase B substrates Azocoll and Hide Powder Azure B grade were obtained from Calbiochem. All other chemicals were obtained from Merck.

3. Results and discussion

Azocoll [5,15] and Hide Powder Azure [5] had been reported as proteinase B substrates and no other enzyme capable of cleaving these substrates has been found as yet in yeast. Thus Petri plates containing one of these substrates embedded in agar should allow detection of proteinase B activity in yeast colonies: A clearing zone around them should appear

owing to hydrolysis of the substrate particles by proteinase B, provided that solutions could be found for the following problems: (1) As proteinase B is an intracellular enzyme located in the vacuole [2,3] the enzyme protein must leak out of the cell. This problem was overcome by using a cell lysis mutant as starting strain. (2) After rupture of the vacuole, proteinase B combines with a specific inhibitor protein localized in the cytosol [2,3] to form an inactive complex. Conditions on the Petri plate leading to inhibitor removal are required. Saheki et al. [13] found that proteinase B in crude extracts could be activated by adjusting the pH to around 5, leading to partial activation of a second endoproteinase present – proteinase A – , that inactivates proteinase B inhibitor. Jones [17] reported 0.26% sodium dodecyl sulfate (SDS) as an activating agent of proteinase B.

A sample of the temperature sensitive cell lysis strain 233 was incubated at 37°C on Petri plates containing Hide Powder Azure or Azocoll and tested both at pH 5 and in the presence of 0.26% SDS. In both cases there was clearing owing to proteinase B action. For mutant screening the following conditions were found to be most satisfactory: One Petri plate contained about 15 ml of growth-promoting solid complete medium (YPD) supplemented with adenine and uracil as described under materials and methods, 0.05% sodium dodecyl sulfate and 10 mg/ml Hide Powder Azure. This will be referred to as the screening medium.

Isolation of proteinase B mutants proceeded as follows: After mutagenesis of strain 233, cells were plated on Petri plates containing complete medium supplemented with adenine and uracil and grown for 4–6 days at 23°C. Then cells were replica plated onto the screening medium and incubated one to three days at 37°C. Colonies not showing a halo were picked from a YPD master plate incubated at 23°C. From about 4000–5000 colonies possible mutants were picked, restreaked and again tested for Hide Powder hydrolysis at 37°C on the screening medium. 28 colonies did not show any clearing and were subjected to biochemical analysis. Crude extracts of seven of these colonies turned out to be absolutely devoid of proteinase B activity when tested using either the pH 5.2 activation procedure of Saheki et al. [13] or when preincubated 4–6 h at 25°C with 0.65% sodium dodecyl sulfate, a concentration that was found to be optimal under our conditions (tab. 1). Figure 1 shows the behaviour of four of the proteinase B mutants and the original strain 233 on a Hide Powder screening plate. To be certain that absence of proteinase B activity in the mutants was not caused by a lack of proteinase A, the enzyme that destroys proteinase B inhibitor during the pH 5.2 treatment [13], proteinase A activity was also measured. Table 1 shows, that

Table 1
Proteinase activities of cell lysis strain 233 and mutants isolated

Strain	Specific activity (units/mg)					
	Proteinase B		Proteinase A		Carboxypeptidase Y	
	pH 5.2	SDS	pH 5.2	SDS	pH 5.2	SDS
233	39×10^{-3}	34×10^{-3}	2.1	1.6	6.1×10^{-3}	6.5×10^{-3}
HP 58	$<0.2 \times 10^{-3}$	$<0.2 \times 10^{-3}$	2.1	1.4	3.1×10^{-3}	2.7×10^{-3}
HP 81	$<0.2 \times 10^{-3}$	$<0.2 \times 10^{-3}$	1.4	0.8	3.7×10^{-3}	3.7×10^{-3}
HP 163	$<0.2 \times 10^{-3}$	$<0.2 \times 10^{-3}$	2.2	1.2	4.7×10^{-3}	4.0×10^{-3}
HP 174	$<0.2 \times 10^{-3}$	$<0.2 \times 10^{-3}$	0.7	0.7	3.8×10^{-3}	0.7×10^{-3}
HP 232	$<0.2 \times 10^{-3}$	$<0.2 \times 10^{-3}$	1.9	2.4	5.2×10^{-3}	3.9×10^{-3}
HP 297	$<0.2 \times 10^{-3}$	$<0.2 \times 10^{-3}$	1.7	0.8	3.4×10^{-3}	2.8×10^{-3}
HP 333	$<0.2 \times 10^{-3}$	$<0.2 \times 10^{-3}$	1.2	1.1	3.5×10^{-3}	2.8×10^{-3}

Activity was measured after two different proteinase activation procedures – pH 5.2 treatment and 0.65% SDS activation – as outlined in section 3. Tests are described in section 2. During pH 5.2 activation procedure proteinase B activity was followed up to 160 h to detect any enzyme activity. Microbial growth in crude extracts was inhibited by 10^{-3} M NaN_3

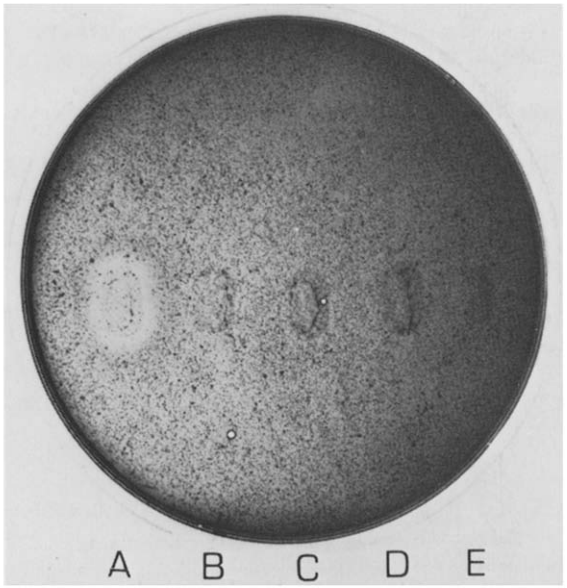

Fig.1. Petri plate test for Hide Powder clearing ability of strain 233 and mutants lacking proteinase B activity. (A) cell lysis strain 233; (B) HP 58; (C) HP 81, (D) HP 163; (E) HP 232. Test was performed as outlined in section 3.

Table 2
Proteinase B-inhibitor activities of cell lysis strain 233 and seven mutants lacking proteinase B activity before and after pH 5.2 treatment for proteinase B activation

Strain	Specific activity (units/mg) proteinase B inhibitor	
	Before pH 5.2 treatment	After pH 5.2 treatment
233	1.2	0.025
HP 58	0.6	0.012
HP 81	0.8	0.048
HP 163	1.5	0.010
HP 174	1.2	0.012
HP 232	1.0	0.010
HP 297	1.4	0.008
HP 333	1.7	0.042

Proteinase B-inhibitor activity was measured as outlined in section 2. Inhibitor activity was followed up to 160 h during pH 5.2 incubation. Boiled extracts were prepared according to [15] and protein values of these extracts were taken for inhibitor level calculation. As protein in boiled extracts is reduced 10- to 12-fold over protein in crude extracts, for comparison with proteinase activity, inhibitor units have to be devided by this factor

proteinase A activity and also carboxypeptidase Y activity is present in all mutants. Other possible explanations for the absence of proteinase B activity in these mutants are: (1) that the concentration of proteinase B inhibitor has been increased; or (2) that some mutation in the proteinase B–inhibitor protein complex has been produced and that this leads to failure to inactivate the inhibitor protein. These possibilities were tested by measuring inhibitor levels before and after pH 5.2 treatment. It was found that the mutants show no significant change in the level of proteinase B inhibitor compared to the wild type strain 233 and that proteinase B inhibitor can be inactivated in all mutants (table 2). Thus absence of proteinase B activity cannot be due to some mutation affecting inhibitor levels or the inhibitor protein. Furthermore, none of the mutants showed petite phenotype, that, in some cases, was found to be connected with reduced proteinase levels [18].

All seven mutants lacking proteinase B activity are able to grow normally and show no aberrant behaviour. However, cells bearing mutations affecting the budding event might be expected to show an abnormal phenotype [19]. Thus one might be tempted to conclude that proteinase B is not involved in a vital role of the budding event, as is the activation of chitin synthetase proposed by Cabib [4] and that another mechanism is probably responsible for this activation. As measured in one of the mutants (HP 163) also glucose inactivation of cytoplasmic malate dehydrogenase activity proceeds in the same way as in the wild type, indicating that proteinase B might not be involved in the primary inactivation event.

One might raise three possible objections to our conclusions: (1) The residual traces of proteinase B inhibitory activity in all strains even after prolonged pH 5.2 activation procedure (table 2) are roughly about 1/20 to 1/100 of the wild type proteinase B activity. This residual proteinase B activity might still be complexed and thus undetectable. (2) The mutation in every one of the mutants might affect only binding or hydrolysis of Azocoll and Hide Powder Azure of proteinase B, leaving its activity against in vivo substrates intact. (3) There might exist other enzymes not able to cleave the synthetic substrates Azocoll and Hide Powder but exhibiting overlapping specificity with proteinase B with respect to in vivo

substrates. We hope to evaluate these possibilities by further genetic and biochemical analysis of the isolated mutants.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft. The help of Dr C. Cooper during preparation of this manuscript and the expert assistence of Mrs L. Kötschau are gratefully acknowledged.

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