Volume 283, number 1, 78–84 FEBS 09693 May 1991

ADONIS 0014579391004024

The proteinase yscA-inhibitor, I^{A}_{3}, gene

Studies of cytoplasmic proteinase inhibitor deficiency on yeast physiology

Peter Schu[2] and Dieter H. Wolf[1]

[1] *Institut für Biochemie der Universität Stuttgart, Pfaffenwaldring 55, D-7000 Stuttgart 80, Germany* and [2] *Biochemisches Institut der Universität Freiburg, Hermann-Herder-Str. 7, D-7800 Freiburg, Germany*

Received 7 March 1991

The gene of the proteinase yscA inhibitor I^{A}_{3}, PAI3, of the yeast *Saccharomyces cerevisiae* was isolated by oligonucleotide screening of a genomic DNA library and sequenced. The gene codes for a single protein of 68 amino acids. The structural PAI3 gene was deleted in vitro by oligonucleotide-site-directed mutagenesis. The mutated allele was introduced via homologous recombination into the genome of wild-type yeast and into the genome of a yeast mutant, which lacks the second cytoplasmic proteinase-inhibitor, I^{B}_{2}. The deficiency of either or of both inhibitors has no effect on the cell viability under various physiological conditions. The inhibitor mutants, however, show an increase in the general in vivo protein degradation rate. The I^{A}_{3} mutant has a 2–3-fold increased protein degradation rate in the first 6 h after a shift from rich medium onto starvation-medium, whereas the I^{B}_{2} mutant shows a constantly increased degradation rate of 20–50% under the same conditions. The inhibitor double null mutant has the same protein degradation rate as the I^{A}_{3} null mutant. These results suggest an in vivo interaction between the vacuolor endopeptidases and their cytoplasmic inhibitors.

Proteolysis; Proteinase inhibitor; I^{A}_{3}; I^{B}_{2}; Null mutant; *Saccharomyces cerevisiae*

1. INTRODUCTION

The vacuole (lysosome) of the yeast is an acidic compartment, which contains multiple hydrolases (see reviews [1–4]). It is also a storage organelle for several compounds like phosphate, amino acids and calcium. The budding yeast *Saccharomyces cerevisiae* contains 1–2 large vacuoles per cell, which undergo multiple fusion and fragmentation processes during cell growth and sporulation (for review, see [5]).

The two vacuolar endopeptidases proteinase yscA and yscB are essential enzymes for protein degradation during vegetative growth and, most pronounced, under starvation conditions ([6], for review see [1–4]). Proteinase yscA is even vital for cell viability under starvation conditions [6]. The peptidases proteinase yscA, proteinase yscB and carboxypeptidase yscY are necessary for the differentiation process of sporulation [6].

The cytoplasm of the yeast *Saccharomyces cerevisiae* contains the peptide inhibitors, I^{A}_{3}, I^{B}_{2} and I^{C}, specific for the vacuolar endopeptidases proteinase yscA, proteinase yscB and carboxypeptidase yscY, respectively [7,8]. The activities of the cytoplasmic proteinase inhibitors, I^{A}_{3} and I^{B}_{2}, and of the vacuolar proteinases, yscA and yscB, are expressed in parallel under all conditions tested [9–12].

No proteinase activities can be detected in cell extracts of wild-type yeast due to the formation of the enzyme/inhibitor complexes. The proteinase activities can be recovered by incubation at pH 5 or in the presence of detergents [13–16]. The inhibitors were found in the cytoplasmic fraction and only traces were found in the microsomal fraction [7]. The proteinase inhibitors are small and heat stable proteins (I^{A}_{3}: 7.6 kDa; I^{B}_{2}: 8.5 kDa), which contain no disulphide bonds [17–20]. Little is known about the function of the cytoplasmic proteinase inhibitors.

The intracellular distribution of the proteinases and their inhibitors, as well as the observation that they are expressed under identical physiological conditions, led to the hypothesis that the inhibitors protect the cell against unwanted proteolytic activities of the vacuolar enzymes in the cytoplasm. Leakage events were thought to be possible due to the observed fragmentation and fusion processes of the vacuole [8]. We have previously shown that the deletion of the structural gene of the I^{B}_{2} inhibitor does not lead to an altered physiology of the mutant, although the in vivo protein degradation rate is 20–50% increased under starvation conditions [12]. To learn more about the in vivo function of these cytoplasmic proteinase inhibitors, we constructed a mutant deficient in the proteinase yscA inhibitor I^{A}_{3} and one

Correspondence address: D.H. Wolf, Institut für Biochemie der Universität Stuttgart, Pfaffenwaldring 55, D-7000 Stuttgart 80, Germany. Fax: (49) (711) 685-4392

 Published by Elsevier Science Publishers B.V.

lacking both inhibitors, I^{A}_{3} and I^{B}_{2}. Here we report the isolation of the I^{A}_{3} gene, the construction of the various null mutants and the analysis of the physiology of the mutants.

2. MATERIALS AND METHODS

2.1. E. coli and yeast strains

E. coli strains: JM109 (*reA1, endoA1, gyrA96, thi⁻, hsdR17, supE44, relA1* (*lac, pro*) *F'traD36, proAB, lacI^q, lacZ, ΔM15*) [21]; RZ1032 (*dut⁻, ung⁻*) and BMH71 (*mutL*) [22,23]. Yeast strains: YS18 (MATα, ura3Δ5, his3-11, leu2-3, -112, can^R) [24] and YPS19 (MATα, ib2:URA3, ura3Δ5, his3-11, leu2-3, -112, can^R) [12].

2.2. Media

For growth of *E. coli* standard LB (Luria-Bertani) and TY (modified LB) media were used [25]. Ampicillin was added to 50 mg/l, if required. For cultivating yeast the following media were used [26]: YPD complete medium contained 2% glucose, 1% yeast extract and 2% peptone; MV-mineral medium consisted of 2% glucose, 0.1% yeast extract, 0.67% yeast nitrogen base without amino acids, and supplements required by auxotrophic strains. K-Acetate medium contained 1% acetate and 0.17% yeast nitrogen base without ammonium sulfate. When these media were used as solid media 2% agar was added. All ingredients were from Difco Laboratories (Detroit, MI, USA).

2.3. Chemicals

Enzymes were obtained from Bethesda Research Laboratories Inc. (Eggenstein, Germany) or Boehringer Mannheim (Mannheim, Germany). [α-^{32}P]dATP, [^{35}S-thio]dATP, [γ-^{32}P]dATP and [^{3}H]leucine were purchased from Amersham Buchler GmbH (Braunschweig, Germany). Ampicillin, hemoglobin, amino acids, adenine and uracil were from Sigma (Deisenhofen, Germany). Azocoll was from Calbiochem (Frankfurt, Germany). All other chemicals were of highest purity available.

2.4. Molecular cloning

Procedures followed standard protocols [25,26]. Bacteria were transformed by using the $CaCl_2$ protocol [27]. Yeast was transformed using the Li-acetate method [28].

2.5. Isolation, sequencing and deletion of the PAI3 gene

The genomic DNA library of *Saccharomyces cerevisiae* S288c (α, *mal⁻*) on the centromer plasmid pCS19 [24] was used to isolate the structural gene of I^{A}_{3}. Two sets of oligonucleotides corresponding to amino acids 1–6 and 29–34 [18] (see Fig. 1, 17mer, 64 oligonucleotides in each mixture) were used after labelling with ^{32}P to screen the library of 5000 clones. The DNA fragment, which hybridized with both oligonucleotide-mixtures, was subcloned into pEMBL19 [29]. DNA sequencing was done according to Sanger [30].

The null mutant of I^{A}_{3} was constructed by replacing the chromosomal gene with an allele mutated in vitro [31,32]. The structural PAI3 gene was deleted by site-directed mutagenesis and selection for the mutated DNA was done according to Kunkel [22,23]. The exact removal of the structural gene was demonstrated by sequencing. After insertion of the LEU2 gene [33] into the mutated DNA, yeast strains YS18 [24] and YPS [12] were transformed with 5 μg DNA of the linearized construct and the transformants were selected on MV-plates without leucine. Clones were tested for inhibitory activity against purified proteinase A [15,34].

2.6. Hybridisation of genomic DNA

Chromosomal yeast DNA was digested with *Hin*dIII, electrophoretically separated on a 0.7% agarose gel and hybridized in the dried gel with a ^{32}P-labelled oligonucleotide (17mer, bases −70 to −54), which primed in the promoter region of I^{A}_{3} [35].

2.7. Mating type switch

The MATα mating type of the wild-type strain YS18 was switched to the MATa mating type by transformation with the HO gene [36,37] on the plasmid YCp50 [38], which is under the control of the galactose promoter. The transformed strain was grown in liquid YPD-medium, washed twice and transferred into YP-galactose medium. Aliquots of the cell culture were plated onto YPD-agar plates. Mating type was detected after plasmid loss [39].

2.8. Growth of yeast cells, preparation of extracts, enzyme assay and protein determination

Cells were grown in liquid YPD for 16 h and incubated for 24 h at 30°C after transfer into K-acetate medium. Cells were harvested by centrifugation. For measurement of proteinase yscB and yscA activities, cells (1 ml of a 30% suspension) were broken with glass beads (0.5 mm, 200 μl) in Eppendorf tubes by rigid shaking on a Vortex mixer (3 × 2 min, with intermittent cooling on ice). The resulting extracts were cleared by centrifugation. Proteinase yscB was tested according to [40] using the collagen-derivative azocoll as substrate. The colored products were measured spectrophotometrically at 548 nm. Specific activity of proteinase yscB is expressed as $\Delta E \cdot min^{-1} \cdot mg^{-1}$. Proteinase yscA activity was tested according to [15] using acid denatured hemoglobin as substrate. The increasing amount of non-TCA-precipitable peptides during the incubation period were determined by a modified Lowry-assay [34,41]. Tests were done at 30°C for 30 min.

For measurement of the I^{B}_{2} and I^{A}_{3} activities, cells (30% suspension) were heated for 20 min at 95°C and cleared by centrifugation. Inhibitory activities of I^{A}_{3} and I^{B}_{2} were measured against purified proteinase A (Sigma) or purified proteinase yscB respectively [10,15]. Proteinase yscB, purified according to [37], was a gift from U. Weiser. Increasing amounts of boiled cell extracts were added to the proteinase assays and the proteinase activities were compared to those assays without inhibitors.

2.9. Viability under heat-stress and starvation

Cells were grown in YPD medium to diauxic phase at 23°C, washed twice and transferred onto K-acetate medium at 37°C. Cells were incubated for 24 h. Aliquots of the cell culture were spread onto YPD-agar plates and incubated at 30°C. Colonies were counted after 2 and 4 days of incubation.

Protein degradation measurements were done as described in [12].

3. RESULTS

3.1. Isolation and sequencing of the PAI3 gene

We isolated the PAI3 gene by oligonucleotide screening of a *Saccharomyces cerevisiae* S288c genomic DNA library, containing 10–20 kb fragments integrated into the plasmid pCS19 [24]. Two oligonucleotide mixtures, one corresponding to amino acids Met-1 to Gln-6 (see Fig. 1, 17mer, mixture of 64 oligonucleotides) and a second one corresponding to Ala-29 to Ala-34 (see Fig. 1, 17mer, mixture of 64 oligonucleotides) of the published amino acid sequence [18], were used to screen the library of 5000 clones. A 5.5 kb *Hin*dIII fragment found in three clones crosshybridized with the oligonucleotide mixtures. This fragment was cloned into the pEMBL19 [29] plasmid for further characterization. The subcloned DNA fragment contained a 2.2 kb fragment of the gene bank plasmid pCS19 and a 3.3 kb fragment of chromosomal yeast DNA. By the hybridization of various restriction enzyme fragments with the oligonucleotide mixtures, the structural gene was found to be

```
-111                                                        -60
CTAGAATTATCTATATAACGGTAAAAAGAATAAAACTCTATTCTAGTTCTCG
                                                             -8
CCATTTACCTTGACTTTAATGAACCAATAAAAGAAATTTCTACAACCAAGAC
          1                                                  33
ATCCAGA ATG AAT ACA GAC CAA CAA AAA GTG AGC GAA ATA
        Met Asn Thr Asp Gln Gln Lys Val Ser Glu Ile
        1                                                    11

                                                            72
TTT CAG AGC TCA AAG GAA AAA TTG CAG GGC GAT GCA AAG
Phe Gln Ser Ser Lys Glu Lys Leu Gln Gly Asp Ala Lys
                                                            24

                                                           111
GTA GTG AGT GAC GCT TTT AAG AAA ATG GCT AGT CAA GAC
Val Val Ser Asp Ala Phe Lys Lys Met Ala Ser Gln Asp
                                                            37

                                                           150
AAG GAC GGC AAG ACT ACC GAT GCT GAT GAA AGT GAA AAA
Lys Asp Gly Lys Thr Thr Asp Ala Asp Glu Ser Glu Lys
                                                            50

                                                           189
CAC AAC TAT CAA GAG CAA TAC AAC AAG CTC AAA GGG GCG
His Asn Tyr Gln Glu Gln Tyr Asn Lys Leu Lys Gly Ala
                                                            63

                      207                                  234
GGG CAT AAG AAG GAG TAG CTCTGTTGTCCTACTATCGATTATTTG
Gly His Lys Lys Glu ***
                    68
                                                           286
TGCCAGCGGCTCTGTTAGGTTGAGGGTGGAAGTGTAGAAAGCAGACCTATT
TGCCAGCGATACATGACCACGCTC 310
```

Fig. 1. Sequences of the I^{A}_{3} inhibitor. The TATA-box motif is underlined. Amino acids encoded by rarely used codons are in bold letters.

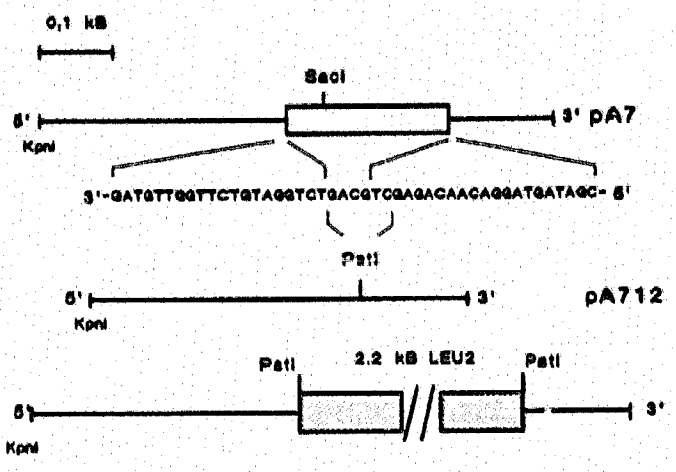

Fig. 2. Deletion of the structural PAI3 gene (box) of the inhibitor (pa7). Hybridisation with the mutagenesis oligonucleotide, with sequences complementary to the promotor- and terminator-regions of the gene. In between these sequences the *Pst*I-recognition site was introduced. The *Pst*I-site of the mutated gene (pA712) was used to insert the LEU2 gene as the auxotrophic marker for the gene replacement experiment.

localized near the *Kpn*I-site of a 2 kb *Hin*dIII/*Kpn*I fragment. Overlapping sequences of both DNA strands of this region were sequenced according to Sanger [30]. We found an open reading frame of 204 nucleotides, starting about 240 nucleotides after the *Kpn*I-site. A putative TATA-box motif is located at position −99 (Fig. 1). The amino acid sequence deduced from the nucleotide sequence is identical with the published primary structure of the protein, which had been determined by amino acid sequencing [12].

3.2. *Construction of I^{A}_{3}- and I^{A}_{3}/I^{B}_{2} double-mutant* strains

The structural PAI3 gene was deleted in vitro by oligonucleotide-site-directed mutagenesis [22,23]. The oligonucleotide consisted of the sequences −20 to −1 and 205 to 226 of the promoter- and the terminator-region of the gene (Figs. 1 and 2). Between these sequences, a *Pst*I-site was introduced, allowing the subsequent insertion of a 2.2 kb *Pst*I-fragment of the LEU2 gene [33] (Fig. 2). The last two bases of the *Pst*I recognition-site are identical with the last two bases of the stop codon. By that, the mutagenesis oligonucleotide contained 45 nucleotides instead of 47 nucleotides. The structural gene was deleted on a 3 kb *Hin*dIII/*Xba*I-fragment of the chromosomal yeast DNA, that had been subcloned into the pEMBL19 [29] plasmid. Mutation was done according to Kunkel [22,23]. The proper removal of the PAI3 gene was confirmed by sequencing. A 2.23 kb *Pst*I-fragment of the LEU2 gene was inserted in the newly generated *Pst*I recognition-site (Fig. 2). This construct was used for the mutation of the wild-type yeast strain and the I^{B}_{2} inhibitor null-mutant.

To study the function of the cytoplasmic inhibitors we introduced the pai3::LEU2 allele into the genomes of the wild-type strain YS18 [24] and of the I^{B}_{2} inhibitor null-mutant YPS19 [12] by gene replacement via homologous recombination [32]. After transformation of the yeast strains YS18 and YPS19 with the linearized LEU2-containing *Hin*dIII/*Xba*I-fragment, clones were selected on the basis of leucine prototrophy.

Ten of the 200 colonies of each transformation experiment were used for the test of the stability of the LEU2 prototrophic phenotype. Seventy percent showed a stable phenotype, which indicates the integration of the mutated allele. Six clones of each mutation were grown on YPD complete medium to the stationary growth phase, where the inhibitory activity is high in wild-type cells, and the cell extracts were tested for inhibitory activity against proteinase A (see section 2). None of these clones showed inhibitory activity (Fig. 3). Subsequent studies were done with the I^{A}_{3} mutant clone named YPS82 and with the I^{A}_{3}/I^{B}_{2} double-mutant clone named YPS95.

The introduction of the mutated allele into the genomes of the yeast strains solely at the PAI3 locus was demonstrated by the hybridization of chromosomal DNA restriction enzyme fragments of the wild-type strain and of the I^{A}_{3}- as well as the I^{A}_{3}/I^{B}_{2}-mutant (see section 2). Deletion of the 204 nucleotide coding region of the *Hin*dIII/*Xba*I fragment and the subsequent insertion of the 2.2 kb LEU2 gene leads to a DNA frag-

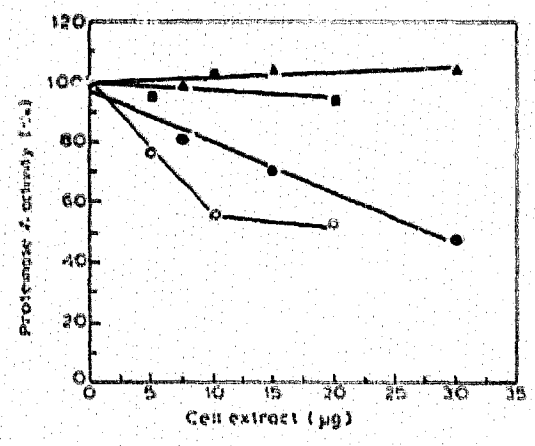

Fig. 3. Inhibitory activities against proteinase A in cell extracts of the various yeast strains. Increasing amounts of boiled cell extract were added to purified proteinase A. Uninhibited proteinase A activity was set to 100%. (A) Wild-type strain YS18 (○) and I^{A}_{3} mutant YPS82 (■). (B) I^{B}_{2} null-mutant YPS19 (●) and the inhibitor double mutant YPS95 (▲).

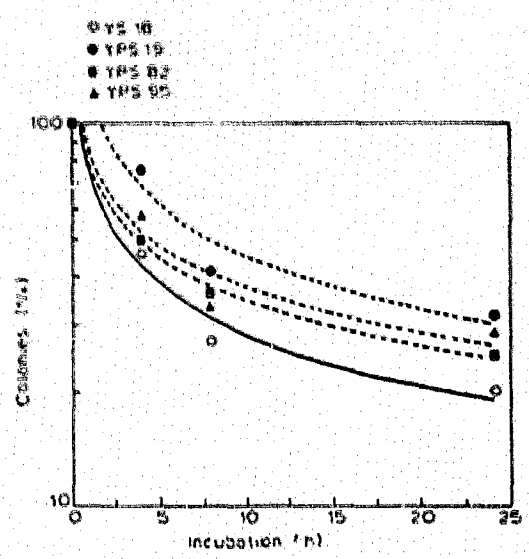

Fig. 4. Viability of the yeast strains under the combination of starvation (K-acetate medium) and heat-stress (37°C). Wild-type strain YS18 (○), I^{A}_{3} mutant YPS82 (■), I^{B}_{2} null-mutant YPS19 (●) and the inhibitor double mutant YPS95 (▲).

ment that is 2 kb larger than the wild-type DNA fragment. The chromosomal DNA of the wild-type strain and the mutant strains were isolated, incubated with *Hin*dIII, electrophoretically separated and hybridized in the dried gel with the ^{32}P-labelled oligonucleotide, corresponding to nucleotides −70 to −54 (see Fig. 1). In the wild-type chromosomal DNA, only a 13.6 kb *Hin*dIII fragment hybridized with the oligonucleotide, whereas in the chromosomal DNA of each mutant, only one 14.5 kb fragment hybridized with the oligonucleotide (not shown). The shift of 2 kb of the hybridized mutant DNA restriction enzyme fragments, compared to the wild-type DNA restriction enzyme fragment, was as expected and demonstrates the deletion of the PAI3 gene and concomitant insertion of the LEU2 gene solely at the PAI3 locus.

3.3. *Growth, sporulation and cell viability*

We looked for effects of the absence of I^{A}_{3} and of I^{A}_{3} and I^{B}_{2} on yeast physiology under various physiological conditions. We tested the growth of cells on the following media: YP-dextrose pH 7 and pH 2.7 at 23°C and 30°C, mineral medium (MV) pH 7 and pH 2.6 and K-acetate medium (media, see section 2). No differences were observed between the mutant strains and the otherwise isogenic wild-type strain (not shown).

Proteinase yscA and proteinase yscB activities are necessary for the process of sporulation, and the activities of both the proteinases and their inhibitors increase in diploid yeast cells under sporulation conditions [6,9,10]. To study the effect of inhibitor absence on sporulation and germination, we constructed isogenic homozygous and heterozygous diploids of the inhibitor single and double null mutants. We switched the mating-type of the original wild-type strain YS18 from MATα to MATa by the use of HO gene (see section 2). The MATa wild-type strain was mated with the MATα mutant strains and after sporulation the mutant spores with a switched mating-type were isolated [43]. These spores were used to generate homozygous diploids, deficient in the I^{A}_{3} inhibitor and in both inhibitors, I^{A}_{3} and I^{B}_{2}. Mating, sporulation and germination of the so-generated strains and spores were not altered compared to the cells of the otherwise isogenic wild-type strain (not shown).

The activities of proteinase yscB and of the proteinase yscB inhibitor I^{B}_{2} increase under heat-stress conditions at 37°C [12,44]. The absence of the I^{B}_{2} inhibitor, however, does not affect the viability of the cells under acute heat-stress conditions [12]. We tested the cell viability of the various inhibitor mutant strains under the combination of heat-stress and starvation conditions. Yeast cultures were grown in glucose medium at 30°C to the late exponential growth phase and then shifted to K-acetate medium without a nitrogen source and further incubated at 37°C. Aliquots of the cell cultures were taken and transferred onto YPD medium agar-plates. Colonies were counted after 2 and 4 days of incubation at 30°C. The inhibitor-deficient mutants show a tendency to stress resistance within the first 8 h after the shift, whereas no difference in the cell viabilities of the strains was observed between 8 and 24 h at 37°C. The tendency to stress-resistance in the first 8 h was most pronounced in the I^{B}_{2} mutant (Fig. 4).

3.4. *Protein degradation*

The proteolytic activities of the vacuolar endopep-

tidases cannot be measured after disintegration of the yeast cell due to the formulation of enzyme/inhibitor complexes. The complexes can be destroyed and the proteolytic activities can be recovered by incubation of the cell extract at low pH in the presence of proteinases [8,16]. In cell extracts of the null mutants deficient in either or both of the inhibitors, the activity of the respective proteinase(s) can be measured immediately and the still-inhibited enzyme in each of the single inhibitor mutants is rapidly activated by the uninhibited proteinase (not shown). We can therefore exclude the existence of heat-labile proteinase inhibitors of proteinase yscA.

Proteinase yscA and proteinase yscB have been shown to be the major unspecific proteinases in yeast cells. The general in vivo protein degradation rate is reduced by about 65-85% in single or double mutants of the vacuolar proteinases under starvation conditions [6]. We tested whether the vacuolar proteinases and their cytoplasmic inhibitors interact in vivo by measuring the general in vivo protein degradation rate in the wild-type strain YS18 and the inhibitor mutants YPS19 (I^B_2 deficient), YPS82 (I^A_3 deficient), YPS95 (deficient in I^B_2 and I^A_3). Cellular proteins were labelled with [^{3}H]leucine in cultures growing in MV-medium to the end of the exponential growth phase. Cells were shifted onto K-acetate medium without a nitrogen source, conditions in which the activities of the proteinases and their inhibitors are high in wild-type cells [9,45], and further incubated up to 24 h in the presence of 5 mM non-radioactive leucine.

The protein degradation rate of the I^A_3 inhibitor mutant YPS82 was, compared to the wild-type strain, 2-3-fold increased in the first 4 h after the shift onto starvation medium. The protein degradation decreased in the following hours, so that the values of the wild-type strain were reached around the fifth hour of incubation (Fig. 5). Under the same conditions the I^B_2 inhibitor mutant showed a constantly increased protein degradation rate of about 20-50% [12]. The mutant deficient in both inhibitors has the same protein degradation rate as the I^A_3 single mutant. Cell viabilities of the wild-type strain and the inhibitor mutant strains are not affected under these conditions (not shown). These results demonstrate that the vacuolar proteinases and their cytoplasmic inhibitors interact in vivo. In our studies with the I^B_2inhibitor mutant YPS19, we could not decide in which cellular compartment, the cytosol or the vacuole, the increased protein degradation takes place [12]. The rapid increase of the protein degradation in the I^A_3 deficient mutant YPS82 in the first hours after the shift onto the starvation medium could be caused by the action of proteinase yscA as the mediator of zymogen activation of vacuolar hydrolases ([46,47,48] for reviews see [2,3]). The observation that the protein degradation rate in the I^A_3, I^B_2 inhibitor double mutant is not simply the sum of the rates observed in the single mutants, but is the same as in the I^A_3 inhibitor mutant, cannot be explained by our present knowledge about vacuolar biogenesis and the regulation of vacuolar protein catabolism.

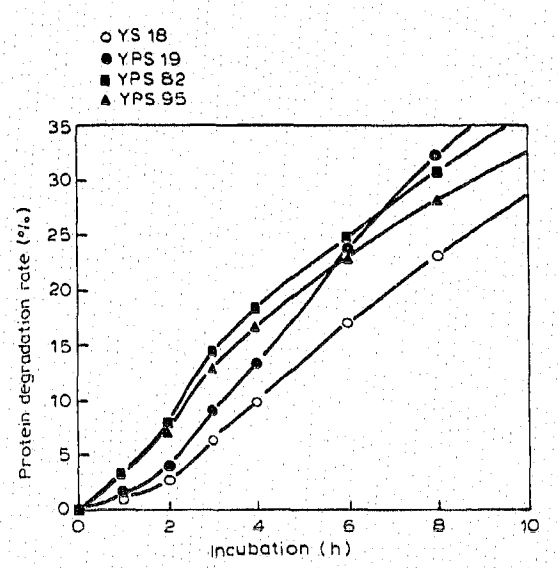

Fig. 5. In vivo protein degradation rate of the yeast strains under starvation conditions (K-acetate medium at 30°C). Wild-type strain YS18 (○), I^A_3 mutant YPS82 (■), I^B_2 null-mutant YPS19 (●) and the inhibitor double mutant YPS95 (▲).

4. DISCUSSION

We have isolated the gene of the proteinase yscA inhibitor I^A_3, PAI3, of the yeast *Saccharomyces cerevisiae*. Sequencing of a subcloned 2 kb *Hin*dIII/*Kpn*I fragment revealed an open reading frame of 204 nucleotides. The amino acid sequence deduced from this nucleotide sequence corresponds with the published primary structure of the protein obtained by protein sequencing [18]. The cytoplasmic proteinase inhibitor I^A_3 is, like the proteinase yscB inhibitor I^B_2 [12], encoded by a single gene and is not part of a precursor protein.

The N-terminal half of the I^A_3 inhibitor protein was found to be the smallest peptide fragment of the protein that shows inhibitory activity against proteinase yscA [18]. The codon usage pattern of the inhibitor gene might reflect the importance of this part of the protein for its inhibitory activity: Sharp et al. [49] found that the amino acids in highly expressed genes are encoded most often by only one or two of the possible base triplets. They concluded that the other, only rarely used codons in those genes did not mutate to the more frequently used codons, because mutations might lead to an exchange of the amino acid. Such a mutation might have a deleterious effect on the protein function, if the amino acid is essential. Most of the codons (78%) of the I^A_3 are of the type required for high expression of the gene. Some codons, encoding amino acids of the N-terminal part of the protein are of the rarely used type

in highly expressed genes. Whereas most of the codons of the I^{A}_{3} mutated to those required for high expression, codons essential for I^{A}_{3} function did not undergo such mutations (see Fig. 1, amino acids and codons are highlighted).

No significant sequence homology of the proteinase yscA inhibitor to functionally related proteins was found in the NBRF and EMBL data banks. A homology of 37% over 51 of the 68 amino acids of this inhibitor to the yeast copper-zink superoxide dismutase was found (not shown). The published purification of the superoxide dismutase includes as the first step the proteolysis of crude cell extract proteins at low pH leaving superoxide dismutase intact [50]. The part of the protein, which shows sequence similarity to the I^{A}_{3} inhibitor might protect the superoxide dismutase against proteolytic degradation under this condition. The I^{A}_{3} inhibitor has no significant homology to the N-terminal pro-sequence of the inactive precursor protein of proteinase yscA [47,48].

Comparison of the amino acid sequence of the proteinase yscB-inhibitor I^{B}_{2} with other protein sequences uncovered a sequence homology of 27% between the entire 75 amino acids of the I^{B}_{2} inhibitor and the pro-sequences of the serine endopeptidases I168 of *Bacillus subtilis* and BPN of *Bacillus amyloliquefaciens* [51]. Proteinase yscB has been shown to be a subtilisin-like enzyme, which shows a 35% homology to the mature bacterial proteinases [52]. The secreted bacillus subtilisins process themselves in an intramolecular reaction, releasing this pro-peptide. The pro-peptide is necessary for the formation of an enzymatically active conformation of the enzyme [53,54]. The I^{B}_{2} inhibitor does not have such a chaperone function, because the in vitro and in vivo activities of proteinase yscB are not reduced in the inhibitor mutants described [12]. The homology between the yeast proteinase yscB-inhibitor I^{B}_{2} and the bacterial serine-proteinase pro-peptides might indicate that the yeast inhibitor evolved from a pro-sequence of a proteinase zymogen. A few amino acid exchanges could lead from a reversible binding, necessary for chaperone function, to a irreversible binding necessary for the inhibitory function. The proteinase yscB precursor protein also contains a N-terminal peptide extension [51], whose primary structure, however, does not show a homology to the pro-sequences of the subtilisins and to the I^{B}_{2} inhibitor.

The yeast inhibitor I^{B}_{2} [20] and the N-terminal pro-sequences of the yeast [52] and the bacterial proteinase [51] have in common a high content of basic amino acids, especially of lysine (I^{B}_{2}: 22.7% basic amino acids, 14.7% lysine; proteinase yscB pro-peptide: 25% basic amino acids, 12.1% lysine; bacterial proteinase pro-peptide: 23.2% basic amino acids, 15.7% lysine). Nothing is known about the importance of lysine for the proteinase-inhibitor binding. The fact that also the yeast inhibitor I^{A}_{3} [18] has a high content of this amino acid (19.1% lysine) might be due to an additional function of the inhibitors unrelated to the specificity of the inhibitor binding. Storage proteins in plants have a high content of lysine and they show significant sequence homologies to proteinase inhibitors [55]. Whether the yeast inhibitors and the amino-terminal pro-peptide of proteinase yscB also fulfill such a storage function for basic amino acids is unknown. It would be consistent with the fact that the inhibitors and the amino-terminal pro-peptide of proteinase yscB (H.H. Hirsch et al., in preparation) are most probably degraded in the vacuole (see section 3.4), which contains most of the cellular basic amino acids.

The entire structural gene of the I^{A}_{3} inhibitor was removed by site-directed mutagenesis and a 2.23 kb LEU2 fragment was inserted at the PAI3 locus as the auxotrophic selection marker for the gene replacement event. This pai3::LEU2 allele was introduced into the genome of the wild-type yeast strain YS18 and of the I^{B}_{2} inhibitor null-mutant strain YPS19 by gene replacement via homologous recombination.

The absence of the inhibitors, either singly or together, did not affect cell growth, mating, germination or sporulation of the mutant strains. Unexpectedly, the inhibitor mutants even showed a tendency to stress-resistance under the combination of heat- and starvational-stress. These results demonstrate that the proteinase inhibitors are not essential for cell viability. The inhibitors could still protect the cell against proteolytic activities of the unspecific vacuolar proteinases in the cytoplasm, however, the presence of these proteinases in the cytoplasm seems to be a rare event and therefore would not affect cell viability.

The fact that the in vivo protein degradation rate is increased in the various inhibitor mutants under starvation conditions, demonstrates an interaction of the cytoplasmic proteinase inhibitors with the vacuolar enzymes in wild-type cells. The constant increase of the protein degradation of 20–50% in the I^{B}_{2} inhibitor mutant [12] and the 2–3-fold increase of the protein breakdown in the first 6 h on starvation medium in the I^{a}_{3} inhibitor mutant, however, do not lead to an altered cell physiology. Together with the previous result, that the half-life of two cytoplasmic enzymes, which are in vitro substrates of proteinase yscB, is not influenced by the inhibitor mutation [12], we hypothesize that the increased protein degradation takes place in the vacuole and not in the cytoplasm.

It has been proposed that the cytoplasmic proteinase inhibitors might protect the cell against unwanted proteolytic activities of the vacuolar enzymes in the cytoplasm, caused by leakage events during the multiple fragmentation and fusion processes of the vacuole [8]. We cannot exclude this function. However, the viability of the inhibitor mutants is not reduced, as one would expect from cells in which unspecific proteinases act uncontrolled in the cytoplasm. Therefore, leakage of the

vacuolar content into the cytoplasm seems to be a rare event. If the cytoplasmic inhibitors reach the vacuole, what function could they have in a compartment, where the proteolytic activities of their counterparts are required? It is known from in vitro results that the proteinases digest themselves [42,56]. The inhibitors can protect the proteinases from auto-digestion and the incompletely inhibited proteinases in the vacuole can still activate each other by degradation of the inhibitors. In that way the inhibitors would maintain a high specific proteolytic activity in the vacuole and they would reduce the proteolytic activities to the required levels. This mechanism would lead to the release of basic amino acids in the vacuole, which contains most of these cellular compounds [5].

Work is in progress to learn more about the organisation of vacuolar protein catabolism, which will hopefully enable us to explain the different effects of inhibitor deficiency on intracellular proteolysis.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt.

REFERENCES

[1] Achstetter, T. and Wolf, D.H. (1985) Yeast 1, 139–157.
[2] Hirsch, H., Suarez Rendueles, P. and Wolf, D.H. (1989) in: Molecular and Cell Biology of Yeasts (Walton, E.F. and Yarranton, G.T. eds) pp. 134–200, Blackie, Glasgow.
[3] Suarez Rendueles, P. and Wolf, D.H. (1988) FEMS Microbiol. Rev. 54, 17–46.
[4] Jones, E.W. (1984) Annu. Rev. Genet. 18, 233–270.
[5] Klionsky, D.J., Herman, P.K. and Emr, S.D. (1990) Microbiol. Rev. 54, 266–292.
[6] Teichert, U., Mechler, B., Müller, H. and Wolf, D.H. (1989) J. Biol. Chem. 264, 16037–16045.
[7] Matern, H., Betz, H. and Holzer, H. (1974) Biochem. Biophys. Res. Commun. 60, 1051–1057.
[8] Holzer, H. and Saheki, T. (1976) Tokai J. Exp. Clin. Med. 1, 115–125.
[9] Betz, H. and Weiser, U. (1976) Eur. J. Biochem. 62, 65–76.
[10] Betz, H. (1975) Biochim. Biophys. Acta 404, 142–151.
[11] Klar, A.J.S. and Halvorson, H.O. (1975) J. Bacteriol. 124, 863–869.
[12] Schu, P., Suarez Rendueles, P. and Wolf, D.H. (1991) Eur. J. Biochem. (in press).
[13] Lenney, J.F. and Dalbec, J.M. (1969) Arch. Biochem. Biophys. 129, 407–409.
[14] Nuñez de Castro, I. and Holzer, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 727–734.
[15] Saheki, T., Matsuda, Y. and Holzer, H. (1974) Eur. J. Biochem. 47, 325–332.
[16] Schwenke, J. (1981) Anal. Biochem. 118, 315–321.
[17] Betz, H., Hinze, H. and Holzer, H. (1974) J. Biol. Chem. 249, 4515–4521.
[18] Biedermann, K., Montali, U., Martin, B., Svendsen, I. and Ottesen, M. (1980) Carlsberg Res. Commun. 45, 225–235.
[19] Bünning, P. and Holzer, H. (1977) J. Biol. Chem. 252, 5316–5323.
[20] Maier, K., Müller, H., Tesch, R., Trolp, T., Witt, I. and Holzer, H. (1979) J. Biol. Chem. 254, 12555–12561.
[21] Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[22] Kunkel, T.A. (1984) Proc. Natl. Acad. Sci. USA 81, 1494–1498.
[23] Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA 82, 488–492.
[24] Sengstag, C. and Hinnen, A. (1987) Nucleic Acids Res. 15, 233–246.
[25] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[26] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Smith, J.A., Seidman, J. and Struhl, K. (1988) Current Protocols in Molecular Biology, Wiley & Sons.
[27] Mandel, M. and Higa, A. (1970) J. Mol. Biol. 53 159–162.
[28] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[29] Denic, L., Cesareni, G. and Cortese, R. (1983) Nucleic Acids Res. 11, 1645–1655.
[30] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[31] Hinnen, A., Hicks, J.B. and Fink, G.R. (1978) Proc. Natl. Acad. Sci. USA 75, 1929–1933.
[32] Rudolph, H., Koenig-Rauseo, I. and Hinnen, A. (1985) Gene 36, 87–95.
[33] Andreadis, A., Hsu, Y.-P., Kohlhaw, G.B. and Schimmel, P. (1982) Cell 31, 319–325.
[34] McDonald, C.E. and Chen, L.L. (1965) Anal. Biochem. 10, 175–177.
[35] Tsao, S.G.S., Brunk, C.F. and Perlman, R.E. (1983) Anal. Biochem. 131, 365–372.
[36] Jense, R.E., Sprague, G.F. and Herskowitz, I. (1983) Proc. Natl. Acad. Sci. USA 80, 3035–3039.
[37] Russel, D.W., Jensen, R., Zoller, M.J., Burke, J., Errede, B., Smith, M. and Herskowitz, I. (1986) Mol. Cell. Biol. 6, 4281–4294.
[38] Pouwels, P.H., Enger-Valk, B.E. and Brammar, W.J. (1988) Cloning Vectors. A Laboratory Manual, Elsevier, Amsterdam.
[39] Chan, R.K. and Otte, C.A. (1982) Mol. Cell. Biol. 2, 11–20.
[40] Saheki, T. and Holzer, H. (1974) Eur. J. Biochem. 47, 621–626.
[41] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[42] Kominami, E., Hoffschulte, H. and Holzer, H. (1981) Biochim. Biophys. Acta 661, 124–135.
[43] Sherman, F., Fink, G.R. and Hicks, J.B. (1986) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[44] Gross, T. and Schulz-Harder, B. (1986) FEMS Microbiol. Lett. 33, 199–203.
[45] López, S. and Gancedo, J.M. (1979) Biochem. J. 178, 769–776.
[46] Mechler, B., Müller, H. and Wolf, D.H. (1987) EMBO J. 6, 2157–2163.
[47] Ammerer, G., Hunter, C.P., Rothman, J.H., Saari, G.C., Valls, L.A. and Stevens, T.H. (1986) J. Cell. Biol. 6, 2490–2499.
[48] Woolford, C.A., Daniels, L.B., Park, F.J., Jones, E.W., Arsdell, J.N.V. and Innis, M.A. (1986) J. Cell. Biol. 6, 2500–2510.
[49] Sharp, P.M., Tuohy, T.M.F. and Mosurski, K.R. (1986) Nucleic Acids Res. 14, 5125–5143.
[50] Steinman, H.M. (1980) J. Biol. Chem. 255, 6758–6765.
[51] Vasantha, N., Thompson, L.D., Rhodes, C., Banner, C., Nagle, J. and Filpula, D. (1984) J. Bacteriol. 159, 811–819.
[52] Moehle, C.M., Tizard, R., Lemmon, S.K., Smart, J. and Jones, E.W. (1987) Mol. Cell. Biol. 7, 4390–4399.
[53] Ikemura, H., Takagi, H. and Inouye, M. (1987) J. Biol. Chem. 262, 7859–7864.
[54] Zhu, X., Ohta, Y., Jordan, F. and Inouye, M. (1989) Nature 339, 483–484.
[55] Hejgaard, J., Rasmussen, S.K., Brandt, A. and Svendsen, I. (1985) FEBS Lett. 180, 89–94.
[56] Dreyer, T., Halkier, B., Svendsen, I. and Ottesen, M. (1986) Carlsberg Res. Commun. 51, 27–41.