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Proteinase yscE of yeast shows homology with the 20 S cylinder particles of *Xenopus laevis*

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Proteinase yscE of the yeast *Saccharomyces cerevisiae* has been compared with the 20 S cylinder particles of *Xenopus laevis*. Both proteins are characterized by a similar group of 10–12 polypeptides with molecular masses ranging between 21 and 38 kDa. Antibodies generated against the 20 S *Xenopus* cylinder particles show cross-reactivity with yeast proteinase yscE subunits. The *Xenopus* particles and yeast proteinase yscE exhibit an identical image in electron microscopy. Both proteins appear as hollow cylinders mostly composed of four stacked annuli. The *Xenopus* 20 S particles exhibit proteolytic activity against the three peptide derivatives known to be substrates of proteinase yscE. The pH optimum for activity and the inhibition spectrum of the proteolytic activities of *Xenopus* 20 S particles and of yeast proteinase yscE are identical. The RNA content of the cylinder particles and of proteinase yscE is below 0.1 RNA chain per molecule. Our data suggest that proteinase yscE from yeast and the 20 S cylinder particles of *X. laevis* are homologous, highly conserved proteins carrying the catalytic character of a peptidase.

Proteinase yscE; 20 S particle; Nucleus; (*Saccharomyces cerevisiae; Xenopus laevis*)

1. INTRODUCTION

Particles sedimenting at about 20 S on a sucrose gradient have been described for a multitude of eukaryotic cells [1–13]. Electron microscopic analysis reveals a high degree of conservation of these particles which were named 'prosomes' [8], 'mini particles' [5], 'ring type particles' [9,10] or 'cylinder particles' [6,7]. In addition, for some of these particle species a rather similar subunit composition has been shown which consists of a 'ladder' of proteins visible on SDS gels ranging from about 19 to 36 kDa in molecular mass. In contrast to the highly conserved morphology and molecular mass range of the subunits, the molecular mass of individual protein subunits varies when different tissues or species are compared (for review see [13]). The intracellular function of the particles is completely unknown. On the basis of in vitro data several suggestions for a function of these particles had been made. They ranged from tRNA synthetase activity or repression of mRNA to tRNA processing [2,8,14].

A significant step towards the elucidation of the in vivo function of the 20 S particles would be their identification in the yeast *Saccharomyces cerevisiae* followed by in vivo genetic and molecular biological analysis to which this organism is easily accessible. From the many peptidases found in *S. cerevisiae* [15–18], proteinase yscE has been shown to be a high-molecular-mass proteinase which upon SDS-polyacrylamide gel electrophoresis exhibited a ladder of protein bands of 35 kDa and less [16]. The similarity of this phenotypic behaviour of proteinase yscE with that of 20 S particles from eukaryotic cells, led us to investigate the relationship of the intensively studied, 20 S cylinder particles of *Xenopus laevis* [6,7] with this proteolytic enzyme from yeast. The results show that the 20 S cylinder particles of *X. laevis*

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have a peptidase activity with nearly identical characteristics as those found for yeast protease yscE, that proteinase yscE consists of a cylinder-type structure identical to the *Xenopus* particles, that both particle species show a similar, but not identical polypeptide composition, and that both proteins exhibit immunological cross-reactivity.

2. MATERIALS AND METHODS

2.1. *Purification of* Xenopus *20 S particles and of proteinase yscE from* Saccharomyces cerevisiae

The 20 S particles of *X. laevis* were purified from total ovary homogenates of this organism as described in [6]. *S. cerevisiae* proteinase yscE was purified using the haploid yeast strain ABYS1 (*a pra1-1 prb1-1 prc1-1 cps1-3 ade*) deficient in the major vacuolar peptidases as outlined in [16].

2.2. *Gel filtration*

The apparent molecular mass of the *Xenopus* 20 S particles was determined by gel filtration of nuclear extracts from *Xenopus* ovaries on a coupled Superose 6 and 12 column system. For reference the following standards were directly mixed with the nuclear extract: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa). The proteins eluted from the column were identified individually by analysis of the fractions on SDS-PAGE.

2.3. *Electron microscopy*

Analysis of purified *Xenopus* particles and *S. cerevisiae* proteinase yscE by negative staining was performed as described in [6]. 20 μl samples were applied to freshly glow-discharged carbon coated grids and negatively stained (2% uranylacetate).

2.4. *Immunological methods*

Antibodies against purified *Xenopus* 20 S particles were raised in guinea pigs. For immunisation, 500 μg of native particles were injected subcutaneously in the presence of Freund's complete adjuvant. Booster injections with 300 μg of *Xenopus* particles each were made on day 21 and 35. Seven days after the last injection blood was taken by heart puncture. The IgG fraction of the serum was obtained by DEAE-cellulose chromatography [19].

Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose sheets essentially as described in [20,21]. Free binding sites on the nitrocellulose filters were blocked by sequential incubations of 30 min with 0.05% Tween in PBS-buffer (137 mM NaCl, 3 mM KCl, 7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and 1% bovine serum albumin in PBS buffer. Filters were then incubated with antiserum (1:150, v/v, diluted in PBS buffer containing 0.1% bovine serum albumin) for 3.5 h and washed by standard protocols including washing solutions which contained 0.1% Triton or 0.4 M NaCl in PBS buffer. The immunoreaction was visualized by the reaction of alkaline phosphatase linked to anti-guinea pig IgG antibody.

2.5. *Proteinase assays*

All tests were done at 30°C. Proteolytic activity against Cbz-Gly-Gly-Leu-4-nitroanilide and Suc-Phe-Leu-Phe-4-nitroanilide was measured as described in [16]. Absorbance of the 4-nitroaniline released was measured at a wavelength of 405 nm. Proteolytic cleavage of Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin was measured in the same buffer (0.1 M Tris-Cl, pH 8.2) as cleavage of the chromogenic substrates. Test volume was 0.4 ml. The amount of fluorescence formed was determined in a fluorescence spectrophotometer (Eppendorf) set at an excitation wavelength of 366 nm and an emission wavelength of between 400 nm and 3000 nm. To determine the number of mol of substrate hydrolyzed, the fluorescence yield of samples was compared to a standard curve prepared with known concentrations of authentic 7-amino-4-methylcoumarin in the same buffer. Activities are expressed in milliunits as the amount of enzyme that catalyzes the release of 1 nmol product/min under the test conditions.

3. RESULTS

3.1. *Morphology and immunological relationship of* Xenopus *20 S cylinder particles and yeast proteinase yscE*

Highly purified preparations of *S. cerevisiae* proteinase yscE and, for comparison *X. laevis* 20 S cylinder particles, were subjected to electron microscopy. Fig.1A shows a survey of the image of the negatively stained protein preparation of proteinase yscE. Typical ring-shaped structures are visible. They are identical to the 20 S *Xenopus* particles described previously [6] with an outer diameter of 10–12 nm and a hollow core of about 4 nm (fig.1B, a and b). As shown for the *Xenopus* particles [6] (fig.1B; b) also rectangular structures of approx. 16 nm length and 12 nm width are visible, which mostly reveal a sub-composition of four plate-like units (fig.1B; a and b). Also other images showing two or three dots or a channel are found in preparations from both organisms. Thus, proteinase yscE is of identical image and size as is found for the *Xenopus* 20 S particles, indicating that the proteins of both organisms are indeed of very similar structure. As a structure a hollow cylinder has been proposed [6].

Gel filtration revealed an apparent molecular mass of about 675 kDa for the *Xenopus* particle (not shown), which is similar to the molecular mass of 600 kDa estimated previously for proteinase yscE [16].

As can be seen in fig.2A, the polypeptide composition of *Xenopus* particles and yeast proteinase yscE are quite similar. *Xenopus* particles show a

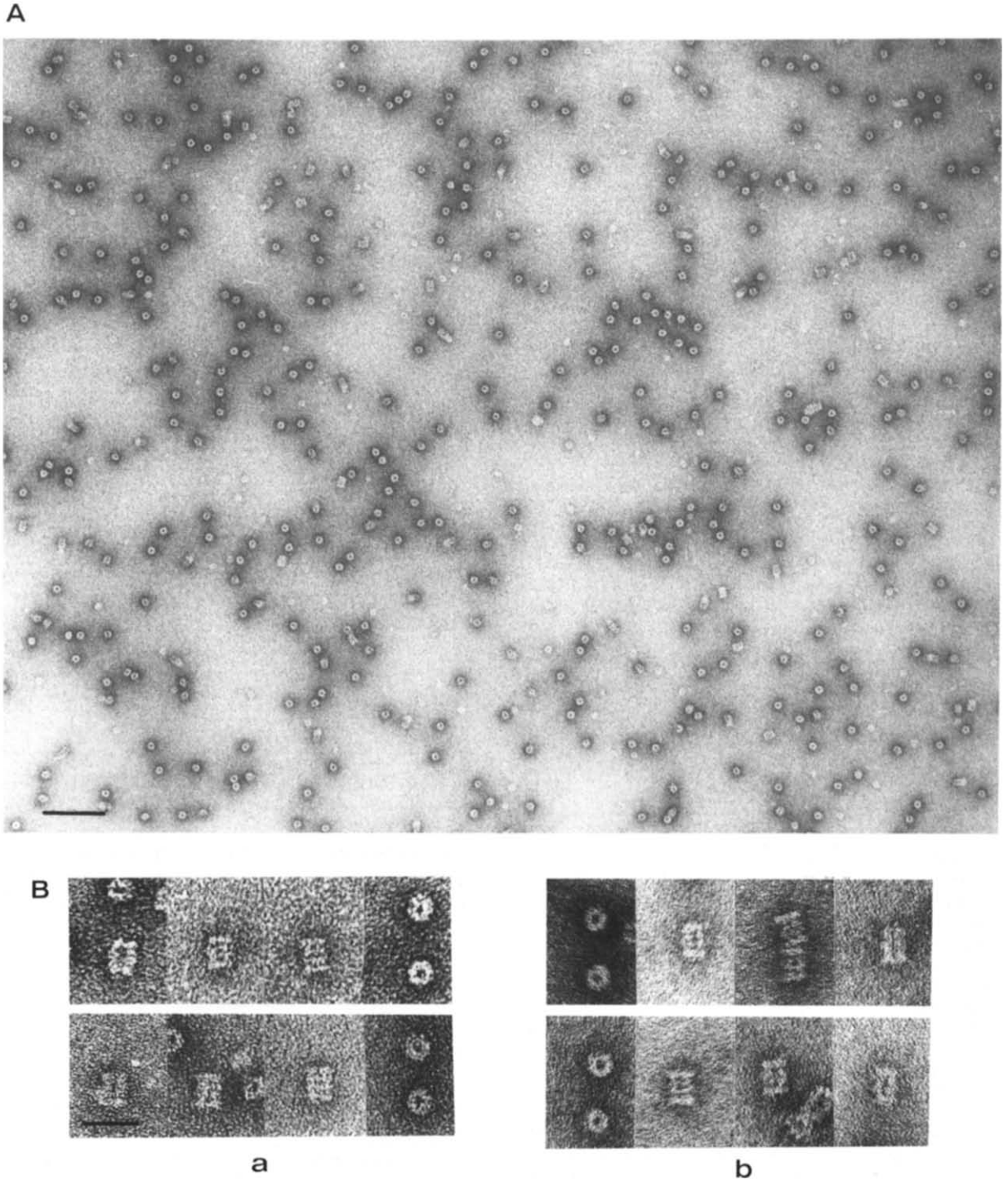

Fig.1. Proteinase yscE from *S. cerevisiae* shows an identical morphology to 20 S cylinder particles from *X. laevis*. (A) Survey electron micrograph of proteinase yscE illustrating the ring- and cylinder-shaped particles. Bar, 0.1 μM. (B) Higher magnification of representative particles from (a) proteinase yscE, (b) 20 S cylinder particles of *X. laevis*. Bar, 30 nm.

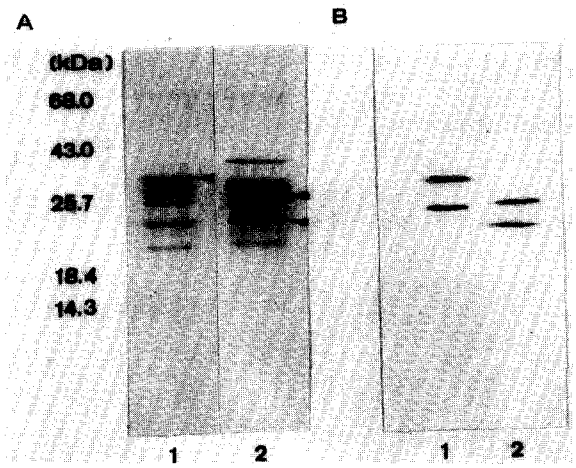

Fig.2. Subunit composition (A) and immunological cross-reactivity (B) of purified 20 S particles of *X. laevis* and proteinase yscE from *S. cerevisiae*. (A) SDS-PAGE (12% gel, according to [34]) of dissociated 20 S particles from *Xenopus* oocytes (2.5 μg) (lane 1) and yeast proteinase yscE (10 μg) (lane 2). Molecular mass markers were bovine serum albumin (68 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25.7 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa). (B) Immunoblot (18% gel, according to [35]) of dissociated 20 S particles from *Xenopus* oocytes (1 μg) (lane 1) and yeast proteinase yscE (25 μg) (lane 2) with polyclonal antibodies against *Xenopus* 20 S particles.

group of 8–10 polypeptides with molecular masses from 21 kDa to 32 kDa, while yeast proteinase yscE is composed of 10–12 polypeptides ranging from 22 kDa to 38 kDa. The molecular masses of the individual subunits of the proteins of both organisms, however, were not identical.

To elucidate the question of if, despite the non-identity of individual subunits of the proteins of both organisms in size, the proteins are nevertheless related, we checked for immunological cross-reactivity of polypeptides within the two proteins. The subunits of *Xenopus* particles and yeast proteinase yscE were dissociated and separated by SDS-PAGE followed by Western blotting. Fig.2B shows the reaction of the separated subunits of both proteins with an antibody directed against the *Xenopus* particles. Two bands of the dissociated *Xenopus* particles react with the antibody directed against the *Xenopus* protein (fig.2B, lane 1), one being identifiable as the 32 kDa subunit (see arrow fig.2A, lane 1). When a considerably higher protein concentration is applied, also two peptide bands of dissociated yeast proteinase yscE react with the *Xenopus* particle antibody (fig.2B, lane 2). Their molecular masses are in the range of 30 kDa and 25 kDa. As freshly prepared batches of the *Xenopus* particles show mainly only the upper antibody cross-reacting polypeptide band of 32 kDa (not shown), we consider the faster moving cross-reacting band to be a degradation product of the slower migrating 32 kDa subunit, which is created during storage of the particles. However, it is obvious that the polyclonal antibody used uncovers homology between subunit polypeptides of *Xenopus* particles and yeast proteinase yscE subunits.

3.2. *Biochemical function of the* Xenopus *particles:* Xenopus *particles carry the proteolytic activity of yeast proteinase yscE*

The identity of the electron microscopic image of proteinase yscE with the *Xenopus* 20 S cylinder particles as well as the antigenic relationship of subunits of the two protein species made it very likely that the *Xenopus* particles also carried the proteolytic activity of yeast proteinase yscE. This is indeed the case: table 1 shows that the *Xenopus* 20 S cylinder particles exhibit proteolytic activity against the same three peptide derivatives Cbz-Gly-Gly-Leu-4-nitroanilide, Suc-Phe-Leu-Phe-4-nitroanilide and Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin, which are substrates of proteinase yscE. The proteolytic activity is associated with the particle protein: specific antibodies raised against the highly purified *Xenopus* particles lead to clearance of the proteinase activity from the incubation mixture (not shown). As found for proteinase yscE [16], the pH dependency of the

Table 1

Xenopus 20 S cylinder particles exhibit proteolytic activity similar to proteinase yscE

Substrate tested	Specific activity (mU/mg)	
	Xenopus particles	Proteinase yscE
Cbz-Gly-Gly-Leu-NA	31	167
Suc-Phe-Leu-Phe-NA	45	81
Suc-Leu-Leu-Val-Tyr-MCA	57	360

Proteolytic activity was measured as outlined in section 2. Between 4 and 5.5 μg of protein were included in the test; NA, 4-nitroanilide; MCA, 7-amido-4-methylcoumarin

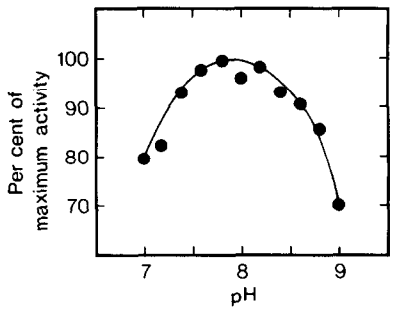

Fig.3. Dependence of the proteolytic activity of the *Xenopus* 20 S particles on pH. 4 μg of *Xenopus* 20 S particle protein was included in the test. Activity was measured against Cbz-Gly-Gly-Leu-4-nitroanilide as outlined in section 2. Buffer was 0.1 M Tris-HCl.

reaction of the *Xenopus* particles with Cbz-Gly-Gly-Leu-4-nitroanilide shows a broad optimum with a maximum between pH 7.6 and pH 8.2 (fig.3). The proteolytic activity of the *Xenopus* 20 S particles is inhibited by $HgCl_2$, 4-hydroxymercuribenzoate, iodoacetamide and chymostatin (table 2), the same inhibitors known to block proteinase yscE activity [16]. The serine proteinase inhibitor diisopropyl fluorophosphate at a concentration of 1 mM does not inhibit the activity of the *Xenopus* particles (table 2), nor proteinase yscE (not shown).

Several reports describe the stoichiometric association of cylinder particles with RNA (for review see [13]), but neither in the 20 S *Xenopus* particles, nor in yeast proteinase yscE was such a stoichiometric relationship found. For both proteins an absorbance ratio at the wavelengths of 280 nm and 260 nm of 1.6 was calculated. Assuming the reported average chain length of 80 nucleotides per particle (for review see [13]), less than 0.1 RNA molecules per 1 particle molecule were present.

Table 2

Effect of inhibitors on the proteolytic activity of the *Xenopus* 20 S cylinder particles

Inhibitor	Final concentration	Activity (% of control)
None		100
$HgCl_2$	0.01 mM	< 1
4-Hydroxymercuribenzoate	0.01 mM	< 1
Iodoacetamide	200 mM	< 1
Phenylmethylsulfonyl fluoride	1 mM	98
Diisopropylfluorophosphate	1 mM	110
EDTA	1 mM	106
Chymostatin	0.1 mg/ml	16
Pepstatin	0.1 mg/ml	101

Proteolytic activity was measured with Cbz-Gly-Gly-Leu-4-nitroanilide as outlined in section 2. Incubation with inhibitors was done for 0.5 h at 30°C. 4 μg of protein were included in the test

4. DISCUSSION

Our results show that proteinase yscE of the yeast *S. cerevisiae* and the 20 S cylinder particles of *X. laevis* exhibit identical electron optical images, have a similar subunit composition with respect to molecular mass range and complexity and share antigenic determinants. Furthermore, the 20 S cylinder particle of *Xenopus* has a proteinase activity. The characteristics of this proteinase concerning substrate specificity, pH optimum for proteolytic activity and susceptibility to inhibitors are identical to yeast proteinase yscE. All these characteristics highly support the fact that *Xenopus* 20 S cylinder particles and proteinase yscE from *S. cerevisiae* are homologous proteins. Both proteins carry a SH group necessary for proteolytic activity. Recently, a report on the detection of 20 S particles from the yeast *S. cerevisiae* was published [12]. Even though this report shows the electron optic image of a few single ring-shaped particles and one cylinder-shaped particle, the authors fail to demonstrate a subunit composition characteristic for proteinase yscE and 20 S particles of other organisms. Only a major band of 26 kDa is visible on SDS gels [12]. Either additional ring-shaped particles exist of different subunit composition than proteinase yscE in *S. cerevisiae* or the preparation investigated [12] was highly contaminated with unrelated proteins. Our findings of the homology of yeast proteinase yscE with the 20 S cylinder particles from *Xenopus* make it very likely that proteinase yscE is equivalent to the 20 S particles of other organisms.

It has been shown very recently that a high-molecular-mass proteinase from mammalian tissues called 'multicatalytic proteinase complex' and 'large alkaline multifunctional protease' or 'proteasome' is homologous with the 19 S 'prosome' particle of mammalian cells and the 19 S ribonucleoprotein from *Drosophila* [22,23] which

themselves belong to the 20 S particle family [12]. It thus appears that the 20 S particle family is a family of high-molecular-mass proteinases ubiquitous from yeast to man. No in vivo function could be assigned as yet to the 20 S particle family (for review see [13]), nor to the proteinases found [16,24–33]. The RNA often found to be associated with the 20 S particles has led to the view that they represent a new type of RNP particle [8,11,12]. Our results do not support this suggestion. The RNA content of the *Xenopus* particle and yeast proteinase yscE are below 0.1 RNA molecules per protein molecule indicative of a non-stoichiometric relationship of RNA and protein in these particles.

The rather conserved structure of the proteinase particles throughout the world of eukaryotic cells investigated so far suggests that the functions of this protein particle are essential to them all. The answer to the question about these functions can only come from mutants defective in the proteinase particle. Yeast is the organism suited best to answer rigorously the questions as it is easily accessible to genetics and molecular biology. We have isolated mutants defective in the activity of proteinase yscE. Studies on one of these mutants shows a defective differentiation (sporulation) process (Escher, C. and Wolf, D.H., in preparation). Studies on the isolation of the genes of the subunit peptides from the proteinase yscE particle are under way in order to disrupt their chromosomal alleles. This will lead us to the answers concerning the function of every individual subunit of this ubiquitous proteinase particle molecule.

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