Biochimica et Biophysica Acta, 384 (1975) 203—214

BBA 67425

PROTEOLYTIC ACTIVITIES IN YEAST

TAKEYORI SAHEKI* and HELMUT HOLZER

Biochemisches Institut der Universität Freiburg im Breisgau and Institut für Biochemie der Gesellschaft für Strahlen- und Umweltforschung, D-78 Freiburg im Breisgau (G.F.R.)

(Received August 26th, 1974)

Summary

Studies on the mechanism and time course of the activation of proteinases A (EC 3.4.23.8), B (EC 3.4.22.9) and C (EC 3.4.12.—) in crude yeast extracts at pH 5.1 and 25° C showed that the increase in proteinase B activity is paralleled with the disappearance of proteinase B inhibitor. Addition of purified proteinase A to fresh crude extracts accelerates the inactivation of the proteinase B inhibitor and the appearance of maximal activities of proteinases B and C. The decrease of proteinase B inhibitor activity and the increase of proteinase B activity are markedly retarded by the addition of pepstatin. Because 10^{-7} M pepstatin completely inhibits proteinase A without affecting proteinase B activity, this is another indication for the role of proteinase A during the activation of proteinase B. Whereas extracts of yeast grown on minimal medium reached maximal activation of proteinases B and C after 20 h of incubation at pH 5.1 and 25° C, extracts of yeast grown on complete medium had to be incubated for about 100 h. In the latter case, the addition of proteinase A results in maximal activation of proteinases B and C and disappearance of proteinase B inhibitor activity only after 10—20 h of incubation. With the optimal conditions, the maximal activities of proteinases A, B and C, as well as of the proteinase B inhibitor, were determined in crude extracts of yeast that had been grown batchwise for different lengths of time either on minimal or on complete medium. Upon incubation, all three proteinases were activated by several times their initial activity. This reflects the existence of proteolytically degradable inhibitors of the three proteinases and together with the above mentioned observations it demonstrates that the "activation" of yeast proteinases A, B and C upon incubation results from the proteolytic digestion of inhibitors rather than from activation of inactive zymogens by limited proteolysis.

* Present address: Department of Enzyme Chemistry, Institute for Enzyme Research, Tokushima University, School of Medicine, Kuramoto-Cho 3, Tokushima 770, Japan.

Introduction

Three different proteinases, usually designated A (EC 3.4.23.8), B (EC 3.4.22.9) and C (EC 3.4.12.—) have been purified and characterized from yeast [1—4]; C has proved useful as a reagent in protein chemistry [4] because it is a carboxypeptidase which will proceed through proline residues. The two tryptophan synthase (EC 4.2.1.20)-inactivating enzymes I and II isolated in our laboratory [5,6] have been shown to be identical or very similar to the proteinases A and B, respectively [7]. Furthermore, it has been shown by Cabib and Ulane [8] and in our laboratory [9] that proteinase B is able to activate pre-chitin synthase by limited proteolysis. Our interest is now directed to the question of the intracellular functions attributed to these proteinases. As a basis for such studies, assay methods for the diffrent proteinases in cells under various physiological conditions, such as different growth stages, are necessary. A general difficulty for determinations of proteinase activities in crude extracts from yeast arises from the fact that after disintegration of the yeast cells for the preparation of a crude extract, proteinase inhibitors which are separated from the proteinases in the intact cells by different subcellular compartmentation, bind firmly to the proteinases, and thereby cause inhibition. The proteinase activity then measured in the crude extract is only the surplus (if any) of the proteinase activity over inhibitory activity. As shown for proteinase B, incubation of crude extracts at pH 5 causes activation of the proteinase [2] by proteolytic inactivation of the firmly bound inhibitor [3]. In the present paper, incubation at pH 5 for proteolytic activation of proteinases has been studied more systematically and used to develop assay methods for proteinases A, B and C in crude yeast extracts.

Materials and Methods

Materials

Pyridoxal 5′-phosphate monosodium salt, L-serine, 4-dimethylaminobenzaldehyde, hemoglobin and toluene were obtained from Merck (Darmstadt, Germany); *N*-acetyltyrosine ethylester and phenylmethanesulfonylfluoride, from Serva (Heidelberg, Germany); glass beads (d = 0.25 mm) and ethanol from Roth (Karlsruhe, Germany); DEAE-Sephadex A-50 from Pharmacia (Uppsala, Sweden); Azocoll from Calbiochem (Los Angeles, U.S.A.). Baker's yeast was purchased from "BÄKO" Bäcker-Einkauf EGmbH (Freiburg, i. Br., Germany). Pepstatin was a generous gift from Professor H. Umezawa. An antibiotic mixture containing 50 mg/ml streptomycin sulfate, 40 000 I.U./ml penicillin G, 10 000 I.U./ml nistatin, and 10 mg/ml neomycin sulfate was kindly provided by Dr A. Hasilik.

Yeast strain and culture

The haploid wild-type strain X 2180-B, originally isolated by R. Mortimer (Berkeley) was used in these experiments. The cells were grown in "complete medium" or in "minimal medium" as described by Katsunuma et al. [5].

Preparation of crude extract

Crude extracts were prepared as described by Katsunuma et al. [5] using a Braun homogenizer (B. Braun, Melsungen, Germany).

Assay of enzyme activities

Tryptophan synthase-inactivating activity. Tryptophan synthase-inactivating activity was measured as described by Saheki and Holzer [7]. Activity is expressed as munits tryptophan synthase inactivated per min per ml.

Proteinase A activity. Proteinase A activity was determined according to the method of Hata et al. [2] as modified by Saheki and Holzer [7] using acid-denatured hemoglobin as substrate. The trichloroacetic acid-soluble product was determined according to the modified Folin colorimetric method of McDonald et al. [10]. Units were defined according to Hata et al. [2].

Proteinase B activity. Proteinase B activity was determined as described by Saheki and Holzer [7] using azocoll as substrate. Activity was expressed as A_{520nm} per min per ml.

Proteinase C activity. Esterolysis of *N*-acetyltyrosine ethylester at pH 8.0 was determined by the pH-stat method as described by Hata et al. [2] using Radiometer Model SBR2/ABU 12/TTT 11/PM26 titration equipment (Copenhagen, Denmark). Since proteinase B has also *N*-acetyltyrosine ethylester-hydrolyzing activity [2], proteinase C activity was calculated using the ratio of *N*-acetyltyrosine ethylester-hydrolyzing activity to azocoll-hydrolyzing activity determined from a purified proteinase B preparation which contained no proteinase C (see also ref. 7), as follows:

$$\text{Proteinase C} = \text{Ac-Tyr-OEt} - 0.98 \times \text{azocoll},$$

where Ac-Tyr-OEt stands for *N*-acetyltyrosine ethylester-hydrolyzing activity and azocoll for azocoll-hydrolyzing activity.

Inhibitory activities against tryptophan synthase inactivase and against proteinase B

Inhibitory activities against inactivase were determined by the method of Ferguson et al. [11] but activity was calculated with linear interpolation as described by Betz et al. [12] (inhibitory units: munits $\cdot$ min^{-1}). Inhibitory activity against proteinase B was determined as described by Betz et al. [12] (inhibitory units: $A_{520nm} \cdot min^{-1}$).

Protein

Protein was estimated by the method of Lowry et al. [13] using crystalline bovine serum albumin as a standard after precipitation with 5% trichloroacetic acid.

Purification of proteinase A

Proteinase A was purified similarly to the method of Hata et al. [2] with the following modifications: 2 kg of baker's yeast in 2 l 0.1 M potassium phosphate, pH 7.0, were homogenized by passing the suspension five times through a Manton-Gaulin homogenizer (Manton-Gaulin Mfg. Co., Everett, Mass.,

U.S.A.) at 500 kg/cm^2. The homogenate was centrifuged at 27 000 × *g* for 30 min, the pH of the supernatant (2300 ml) was adjusted to pH 5.0 with 20% acetic acid, and incubated at 25° C for 22 h with 1 ml of antibiotic mixture per l supernatant. Then the solution was cooled to 5° C and the pH was adjusted to 3.9. After standing for 2 h, it was centrifuged and the pellet was dissolved in 0.1 M potassium phosphate, pH 7.0. The pH was adjusted to 6.5 with 4 M potassium hydroxide and the solution (300 ml) was fractionated with ethanol. The precipitate which appeared after addition of 50% ethanol was collected and dissolved in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M sodium chloride and dialyzed against the same buffer. The dialyzed solution (180 ml) was mixed with 15 g (dry weight) of DEAE-Sephadex A-50 equilibrated with 0.01 M potassium phosphate containing 0.1 M sodium chloride. After standing for 2 h with occasional stirring, the suspension was extensively washed with the same buffer on a Büchner funnel and packed in a column (5 × 14.5 cm). Active fractions were eluted with a linear gradient of sodium chloride from 0.1 M to 0.5 M in 0.01 M potassium phosphate, pH 7, (total volume: 2 l) and concentrated by ultrafiltration using an Amicon UM10 filter.

The concentrated solution (20 ml) was dialyzed against 0.01 M potassium phosphate, pH 7.0, containing 0.1 M sodium chloride and rechromatographed on a DEAE-Sephadex A-50 column (2.5 × 6 cm). Active fractions were concentrated with solid ammonium sulfate in a dialysis bag. The precipitate was collected by centrifugation and redissolved in 0.05 M sodium acetate, pH 4.8.

This preparation which had a specific activity of 1340 units/mg and which contained a proteinase B activity of 0.065 A_{520nm} per min per mg and a proteinase C activity of 2.8 μmol · min^{-1} · mg^{-1} was used as proteinase A for the incubation of crude extract at pH 5.1.

Purification of proteinase B

Proteinase B was partially purified according to the method of Hata et al. [2] with modifications. 2 kg of baker's yeast was plasmolyzed and autolyzed successively at pH 7 and pH 5 as described by Hata et al. [2]. The autolysate was concentrated with 550 g/l ammonium sulfate and dialyzed against 0.01 M potassium phosphate, pH 7.0. The dialyzed preparation was applied to a DEAE-Sephadex A-50 column equilibrated with 0.01 M potassium phosphate, pH 7.0. Active fractions were eluted with gradients of sodium chloride from 0 to 0.4 M, concentrated with 660 g/l ammonium sulfate and dialyzed against 0.01 M potassium phosphate, pH 7.0. This preparation was rechromatographed on a DEAE-Sephadex column and concentrated as described above.

The partially purified proteinase B which had a specific activity of 0.76 A_{520nm} · min^{-1} · mg^{-1} was used for the determination of inhibitory activity against tryptophan synthase inactivase and against proteinase B.

Incubation at pH 5.1

Crude extract was incubated at pH 5.1 and 25° C in order to activate proteinases. The incubation mixture usually contained 1 ml of crude extract, 50 μl of 10% acetic acid, 50 μl of 0.05 M acetate buffer, pH 4.8, and 1 μl of antibiotic mixture. As indicated, various amounts of proteinase A preparation were substituted for 0.05 M acetate buffer.

Results and Discussion

Tryptophan synthase-inactivating activity and the activities of the proteinases A, B and C during incubation of a crude extract from yeast at pH 5.1 and 25° C are shown in Fig. 1. "Tryptophan synthase inactivase" represents the sum of the inactivating action of inactivases I and II, which have been shown to be identical or very similar to proteinases A and B [7]. After about 20 h incubation at pH 5.1, inhibitory activities against proteinase B and against tryptophan synthase inactivase decrease to zero, whereas proteinase B and tryptophan synthase inactivase activities reach a maximum. Therefore, the maximal proteinase B activity which is measured after destruction of the inhibitors represents the latent proteinase B activity in crude yeast extracts.

Proteinase A shows only a small increase in activity during incubation. Whether this indicates a weakly bound inhibitor or a firmly bound inhibitor available only in small amounts cannot be answered at present. Proteinase C increases several fold and reaches a maximum after 20 h of incubation. The

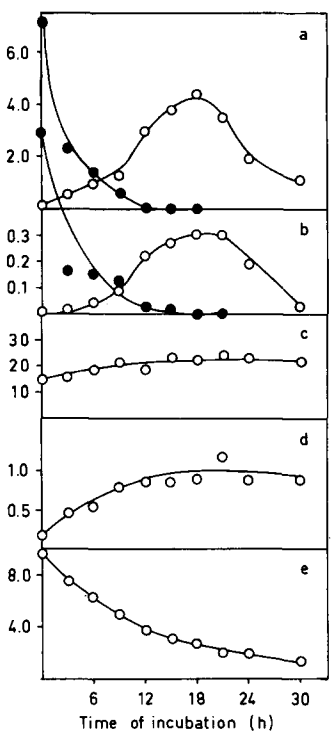

Fig. 1. Activation of proteinases A, B and C in a crude extract of yeast (cultured in minimal medium) by incubation at pH 5.1 and 25° C. Yeast cells were cultured for 23 h at 30° C in minimal medium. Cells were harvested, washed and crude extract was prepared with a Braun homogenizer. 1 ml crude extract (9.6 mg protein) was incubated at 25° C with 50 µl 0.05 M acetate buffer, pH 4.8, 50 µl 10% acetic acid and 1 µl antibiotic mixture. The total volume was 1.1 ml and final pH was 5.1. Tryptophan synthase-inactivating activity, munits · min^{-1} · ml^{-1} (a, o———o); proteinase B activity, A_{520nm} · min^{-1} · ml^{-1} (b, o———o); proteinase A activity, units · ml^{-1} (c); proteinase C activity, µmol · min^{-1} · ml^{-1} (d); and protein concentration, mg · ml^{-1} (e, o———o) were determined. Inhibitory activities against tryptophan synthase inactivase, munits · min^{-1} · ml^{-1} (a, •———•), and against proteinase B activity, A_{520nm} · min^{-1} · ml^{-1} (b, •———•), were determined in boiled samples of the incubations. Ordinates: Units of enzyme activities and inhibitory activities as defined in Materials and Methods.

kinetics of this increase, however, are quite different from those of proteinase B. There might exist another mechanism of activation: Hayashi et al. [4] suggested activation of a pre-enzyme by limited proteolysis. The activated proteinases A and C are fairly stable in the course of further incubation. The maximal activities observed may well approximate the total activities of these enzymes in the crude extract. In contrast, proteinase B activity decreases rapidly after having reached a maximum, and therefore it is difficult to extrapolate the real maximal proteinase B activity present in the fresh crude extract. It will be shown below that addition of proteinase A stabilizes proteinase B, and therefore allows estimation of the maximal activity.

The kinetics of increase and decrease of tryptophan synthase-inactivating activity shown in Fig. 1a are very similar to the changes in the activity of proteinase B (Fig. 1b) and different from those of proteinase A (Fig. 1c). This is in agreement with the earlier observations that the tryptophan synthase-inactivating activity observed in incubated yeast extracts is mainly due to inactivase II, i.e. proteinase B, and only to a small degree to inactivase I, i.e. proteinase A [5,7].

It has been shown by Hata et al. [2] that proteinase A plays a role in the activation of proteinase C. Fig. 2 shows that proteinase A added to an incubation of the crude yeast extract at pH 5.1 and 25° C does not only speed up the activation of proteinase C, but also of proteinase B. The faster decrease of the proteinase B-inhibiting activity during incubation with added proteinase A (see dotted lines in Fig. 2) provides evidence that in this experiment proteinase A acts by proteolytic inactivation of proteinase B inhibitor. This conclusion is

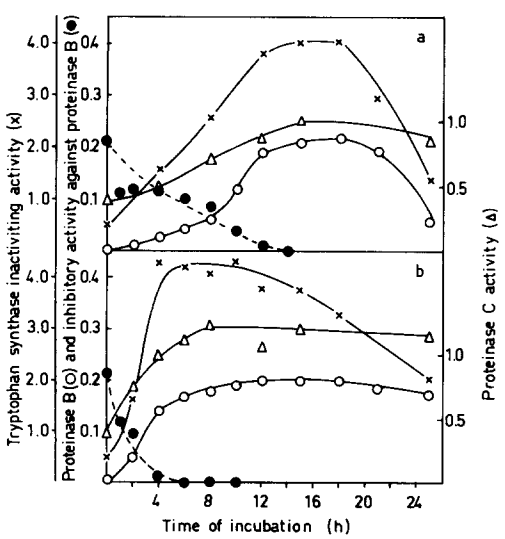

Fig. 2. Effect of added proteinase A on the activation of proteinases B and C and on the decrease of proteinase B-inhibitory activity. 1 ml crude extract (5.7 mg protein) of yeast, cultured for 23 h at 30° C in "minimal medium" was incubated with 50 µl 10% acetic acid, 1 µl antibiotica mixture and with either 50 µl (167 units) proteinase A (b) or 50 µl 0.05 M acetate buffer pH 4.8 (a). Final pH was 5.1, final volume 1.1 ml, temperature 25° C. Tryptophan synthase-inactivating activity, munits · min^{-1} · ml^{-1} (X———X); proteinase B activity, A_{520nm} · min^{-1} · ml^{-1} (○———○); proteinase B-inhibitory activity, A_{520nm} · min^{-1} · ml^{-1} determined in boiled samples (●———●); proteinase C activity, µmol · min^{-1} · ml^{-1} (△———△). For activity units, see Materials and Methods.

TABLE I

EFFECT OF PEPSTATIN ON PROTEINASE A AND B

0.85 μg partially purified proteinase A (1340 units/mg) and 11 μg proteinase B (0.76 $A_{520nm} \cdot min^{-1} \cdot mg^{-1}$) were used as enzymes. Activities of proteinase A and B with and without pepstatin were determined as described in Materials and Methods.

Pepstatin (M)	Remaining activity (%)	
	Proteinase A	Proteinase B
$2.4 \cdot 10^{-9}$	74	—
$6.0 \cdot 10^{-9}$	54	—
$1.2 \cdot 10^{-8}$	42	—
$2.4 \cdot 10^{-8}$	16	—
$1.2 \cdot 10^{-7}$	4	114
$1.2 \cdot 10^{-6}$	—	107
$1.2 \cdot 10^{-5}$	—	113

further supported by the findings of Betz et al. [12] that the two purified proteinase B inhibitors from yeast are rapidly inactivated by incubation with proteinase A.

Pepstatin inhibits acid proteinases as shown by Umezawa et al. [14,15]. Table I shows that concentrations of pepstatin as low as 10^{-7} M inhibit proteinase A completely, whereas proteinase B is not inhibited, even at 10^{-5} M. The experiments shown in Table II demonstrate that pepstatin at concentrations almost completely inhibiting proteinase A inhibits only 17% of the tryptophan synthase-inactivating activity of the incubated crude extract. 77% of the inactivating activity is inhibited by phenylmethanesulfonylfluoride which has been shown in earlier work [7] to be an inhibitor of proteinase B without inhibiting proteinase A. When pepstatin is used together with phenylmethanesulfonylfluoride, 97% of the tryptophan synthase-inactivating activity is inhibited. These results demonstrate that the additive action of proteinases A and B completely explains the observed inactivations of tryptophan synthase and that

TABLE II

INHIBITION OF TRYPTOPHAN SYNTHASE INACTIVATION IN INCUBATED CRUDE YEAST EXTRACT BY PEPSTATIN AND PHENYLMETHANESULFONYLFLUORIDE

Crude extract (4.8 mg/ml protein) of yeast cultured for 40 h in minimal medium was incubated for 17 h as indicated in the legend in Fig. 1. Tryptophan synthase-inactivating activity was measured with and without $5 \cdot 10^{-3}$ M phenylmethanesulfonylfluoride and $7.3 \cdot 10^{-7}$ M pepstatin. In the experiment with phenylmethanesulfonylfluoride, the sample was preincubated for 30 min at 20°C.

Addition at measurement of activity after incubation	Tryptophan synthase-inactivating activity measured after incubation (munits/min per ml)	Inhibition (%)
None	2.4	0
$5 \cdot 10^{-3}$ M phenylmethanesulfonylfluoride	0.55	77
$7.3 \cdot 10^{-7}$ M pepstatin	2.0	17
Phenylmethanesulfonylfluoride + pepstatin	0.13	95

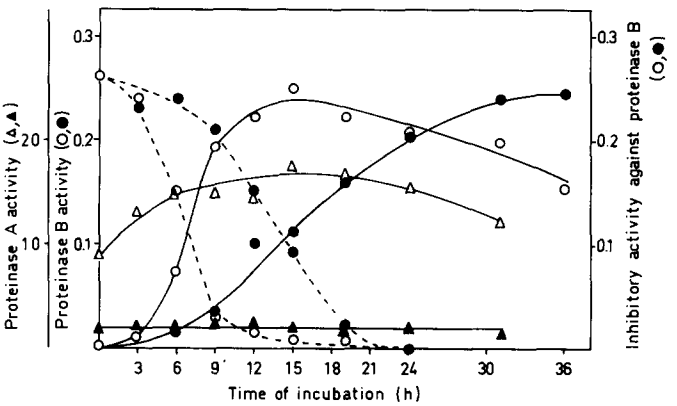

Fig. 3. Effect of pepstatin on the activation of proteinase B and decrease of proteinase B-inhibitory activity. 5 ml crude extract (5.2 mg/ml protein) of yeast cultured for 40 h in minimal medium was incubated with 250 µl 10% acetic acid, 240 µl 0.05 M acetate buffer, pH 4.8; 5 µl antibiotica mixture and 10 µl pepstatin in 10% methanol (final concentration of pepstatin $1.3 \cdot 10^{-6}$ M) at pH 5.1 and 25°C. A control tube contained 10 µl of 10% methanol instead of pepstatin. Proteinase B activity, (○———○) and (●———●), solid line; proteinase A activity, (△———△) and (▲———▲); and inhibitory activity against proteinase B in boiled samples, (○- - - - -○) and (●- - - - -●), dotted line, were measured. Full circles and triangles are with pepstatin, open circles and triangles are without pepstatin. For activity units, see Materials and Methods.

proteinase B is responsible for about 80% of the inactivating activity in activated crude extract. As shown earlier, proteinase C exhibits no tryptophan synthase-inactivating activity [7] and is not inhibited by 10^{-5} M pepstatin (Saheki and Matern, unpublished experiments). The inactivation of tryptophan synthase measures the sum of the activities of the two proteinases A and B. Therefore, in the following part of this paper only the data on selective measurements of proteinases A and B, respectively, are referred to.

As shown in Fig. 3, the addition of pepstatin in a concentration inhibiting proteinase A by more than 90% (see triangles in Fig. 3) slows down the activation of proteinase B during incubation and decreases the rate of disappearance of proteinase B inhibitors.

It can be seen in Fig. 2 that proteinase B activity decreases rapidly after

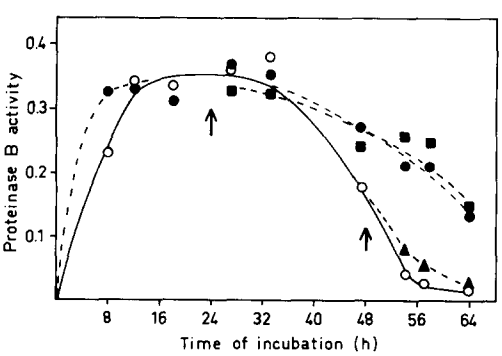

Fig. 4. Stabilizing effect of proteinase A on proteinase B activity during incubation. 1 ml crude extract (9.3 mg protein) of yeast cultured for 48 h minimal medium was incubated at pH 5.1 and 25°C as described in the legend to Fig. 2 without proteinase A (○———○) or with 81 units of proteinase A added at zero time (●———●), at 24 h incubation (■———■) and at 48 h incubation (▲———▲). Ordinate: proteinase B activity, $A_{520nm} \cdot min^{-1} \cdot ml^{-1}$. For activity units, see Materials and Methods.

having reached a maximum. Addition of proteinase A to the incubation mixture slows down the decrease of proteinase B activity. The experiment shown in Fig. 4 demonstrates that proteinase A added after the maximal activity of proteinase B has been obtained also slows down the inactivation of proteinase B during further incubation. In order to measure the maximal activity of proteinase B, it is advisable to add proteinase A to the incubations.

The experiments described above clearly demonstrate the necessity of proteinase A for the activation of proteinases B and C in a crude extract. To be independent from the proteinase A content of the yeast extract to be analyzed for proteinases B and C activity, the addition of proteinase A (free of proteinases B and C) is necessary. The experiments depicted in Figs 5 and 6 have been carried out to establish the optimal conditions for determination of maximal activities of proteinases B and C. Whereas in Figs 2 and 3, extracts from yeast cultured on minimal medium have been studied, Fig. 5 now shows results with an extract from yeast cultured on a complete medium. The upper part of Fig. 5 shows that during incubation without addition of proteinase A, the proteinase B-inhibiting activity disappears very slowly and in agreement with the above-mentioned conclusion that the disappearance of the inhibiting activ-

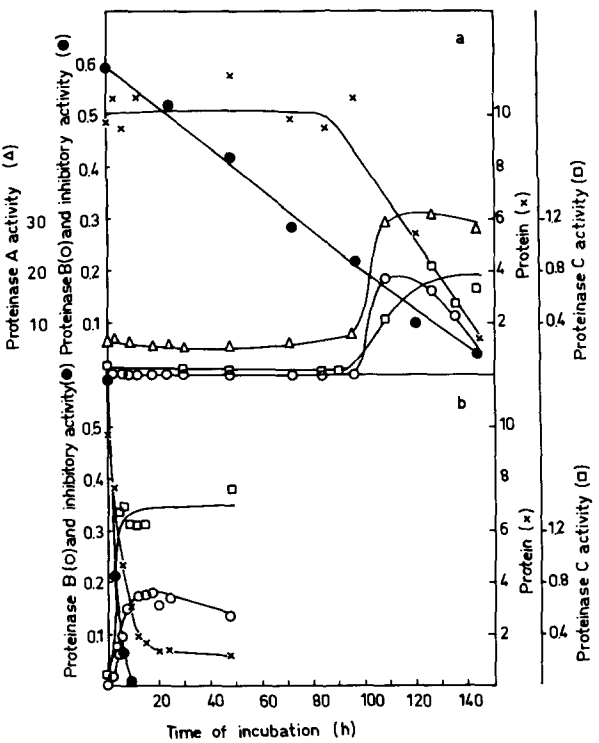

Fig. 5. Activation of proteinases in crude extract of yeast cultured in complete medium by incubation at pH 5.1 and 25° C (a) and effect of added proteinase A on it (b). Crude extract (protein concentration 7.8 mg/ml) of yeast cultured for 48 h in complete medium was incubated as described in the legends to Figs 1 and 2. 162 units proteinase A per 1.1 ml incubation mixture were added in b. Proteinase B activity, $A_{520nm} \cdot min^{-1} \cdot ml^{-1}$ (○———○); proteinase A activity, units/ml (△———△); proteinase C activity $\mu mol \cdot min^{-1} \cdot ml^{-1}$ (□———□); inhibitory activity against proteinase B, $A_{520nm} \cdot min^{-1} \cdot ml^{-1}$, determined in boiled samples (●———●); and protein concentration, mg/ml (X———X), were determined at the indicated times.

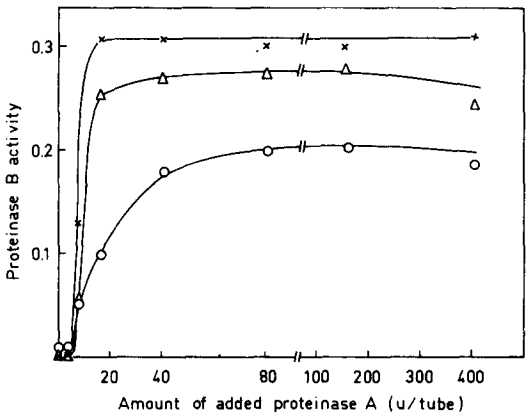

Fig. 6. Effect of added proteinase A on the activation of proteinase B in crude extract of yeast cultured in complete medium. 1 ml crude extract (protein concentration 8.1 mg/ml) of yeast cultured for 48 h in complete medium was incubated with various amounts of proteinase A, 50 µl 10% acetic acid, 1 µl antibiotic mixture, and 0.05 M acetate buffer, pH 4.8, to a total volume of 1.1 ml. Final pH was 5.1, temperature 25°C. Proteinase B activity was determined after 9 h (○———○); 15 h (△———△); and 24 h (X———X) incubation. Ordinate: proteinase B activity, $A_{520nm} \cdot min^{-1} \cdot ml^{-1}$. For activity units, see Materials and Methods.

ity against proteinase B is necessary for appearance of proteinase B activity. It takes more than 100 h until an increase of proteinase B activity is observed. The very long lag period for appearance of an increase of proteinases B and C activity may be due to a masking of proteinase A activity during the incubation at pH 5. The sharp increase of proteinase A activity after 100 h incubation indicates masking by an inhibitor which seems to be also active at pH 3 (used for determination of proteinase A) and is inactivated (perhaps by proteolytic degradation) at prolonged incubation. The sharp decrease of protein concentration in the crude extract beginning after about 100 h incubation corresponds well with the increasing proteolytic activities observed at this time. In the lower part of Fig. 5 it may be seen that after the addition of purified proteinase A to the extract from yeast cultured on complete medium, the time course of appearance of activities of proteinases B and C and disappearance of proteinase B-inhibitory activity and of total protein in the extract are speeded up remarkably. Maximal activities of proteinases B and C are obtained after 20 h incubation. At the present time there is no method available to avoid the extremely long incubation time in order to obtain maximal activities of proteinase A in extract from yeast cultured on complete medium.

Fig. 6 shows the appearance of proteinase B activity during incubation of a crude extract from yeast cultured on complete medium as a function of the amount of proteinase A added. At 24 h of incubation 20 units proteinase A per sample are sufficient to cause maximal activation. Even 400 units proteinase A per sample do not increase the maximum of proteinase B activity.

To determine the maximal activities of proteinases A, B and C in yeast samples, extracts were prepared and incubated under the above-described optimal conditions. The measurements were carried out with yeast that was grown in batch culture for different periods of time either in minimal or in complete medium. The data in Table III revealed two significant findings: (1) Upon

TABLE III

PROTEINASE ACTIVITIES AND INHIBITORY ACTIVITY AGAINST PROTEINASE B IN CRUDE EXTRACTS FROM YEAST GROWN IN BATCH CULTURE

Preparation and incubation of the crude extracts at pH 5.1 and 25°C is described in Materials and Methods. For the measurement of maximal activities of proteinases B and C, 81 units proteinase A were added per incubation mixture containing 1 ml crude extract. Maximal activities of proteinases B and C of crude extracts from minimal medium were obtained after 8 h incubation, and those from complete medium after 16—42 h incubation. Activities are expressed in units per mg protein of fresh crude extract. For definition of activity units, see Materials and Methods.

Culture medium	Culture time (h)	Cell density ($A_{550\,nm}$)	Proteinase A (units/mg)		Proteinase B after incubation** (A_{520nm} $min^{-1} \cdot mg^{-1}$)	Inhibitory activity against proteinase B ($A_{520\,nm}$ $min^{-1} \cdot mg^{-1}$)	Proteinase C after incubation*** ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
			Fresh crude extract	After incubation*			
Minimal medium	9	0.70	0.51	0.96 (42)	0.0035	0.018	0.031
	16	4.3	1.2	2.6 (52)	0.019	0.065	0.055
	24	5.3	1.3	3.3 (23)	0.039	0.12	0.095
	48	7.3	3.1	7.9 (40)	0.061	0.15	0.14
Complete medium	9	2.1	0.40	0.55 (200)	0.0012	0.012	0.043
	16	9.8	1.4	3.4 (98)	0.013	0.15	0.13
	24	14	1.6	4.4 (72)	0.020	0.15	0.12
	48	21	1.3	4.3 (78)	0.023	0.17	0.12

* Hours of incubation in brackets.

** Activity in fresh crude extract was less than 2% of the activity measured after incubation.

*** The increase of proteinase C activity at incubation was variable and ranged between 100 and 500% of the initial activity.

prolonged growth of the cultures both in minimal medium and in complete medium there is a substantial increase of the maximal activities of all three proteinases as well as of the inhibitory activity against proteinase B. The activity increase of proteinase B is particularly striking. (2) The activities of all three proteinases show a marked increase during incubation of the crude extracts. Proteinase A approximately doubles its activity, the activity of proteinase C reaches 2—6-fold of its initial value, the activity of proteinase B increases more than 50-fold. All these changes can be explained by a proteolytic breakdown of inhibitors. Specific inhibitors for proteinase B are already well known [12,16]. Also in the case of proteinase C an inactive proteinase-inhibitor complex has been described [17,18]. We were able to separate from the complex a protein with a molecular weight of 25 000, which specifically inhibited proteinase C [19]. It was also demonstrated that the inhibitor protein is rapidly destroyed by proteolysis by the yeast proteinases A and B [19]. Furthermore two specific inhibitors for proteinase A [20] have been isolated and characterized in our laboratory. The proteinase A inhibitors are rapidly destroyed by proteinase B [20], the proteinase B inhibitors by proteinase A [12], and the proteinase C inhibitor by proteinases A and B [19]. These observations, and the reciprocal time course of the activities of proteinase B and proteinase B-inhibitory activity shown in Figs 1—3, as well as the parallelism of appearance of proteinase A activity (probably by inactivation of the proteinase A inhibitors [20]) with the activation of proteinases B and C, shown in Fig. 5, strongly support the idea that the observed activations of the proteinases A, B and C in incubated ex-

tracts were due to destruction of inhibitors rather than activation of zymogens by limited proteolysis.

The methods described here for the determination of proteolytic activities in yeast are used in our laboratory as a basis for further studies on the influence of growth conditions on the activities of proteinases and inhibitors. Such measurements are, in turn, a necessary prerequisite for further studies on regulatory mechanisms in which proteinases and inhibitors are involved.

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 46. The authors thank Professor H. Umezawa for the generous gift of pepstatin and Drs A. Hasilik and C.-P. Heinrich for help with the preparation of the manuscript. The excellent assistance of Miss D. Montfort, Mrs S. Gräfin zu Eulenburg and Miss E. Conrad are also acknowledged.

References

1 Willstätter, R. and Grassmann, W. (1926) Hoppe-Seyler's Z. Physiol. Chem. 153, 250—282
2 Hata, T., Hayashi, R. and Doi, E. (1967) Agric. Biol. Chem. 31, 150—159
3 Lenney, J.F. and Dalbec, J.M. (1969) Arch. Biochem. Biophys. 129, 407—409
4 Hayashi, R., Moore, S. and Stein, W.H. (1973) J. Biol. Chem. 248, 2296—2302
5 Katsunuma, T., Schött, E., Elsässer, S. and Holzer, H. (1972) Eur. J. Biochem. 27, 520—526
6 Schött, E.H. and Holzer, H. (1974) Eur. J. Biochem. 42, 61—66
7 Saheki, T. and Holzer, H. (1974) Eur. J. Biochem. 42, 621—626
8 Cabib, E. and Ulane R. (1973) Biochem. Biophys. Res. Commun. 50, 186—191
9 Hasilik, A. and Holzer, H. (1973) Biochem. Biophys. Res. Commun. 53, 552—559
10 McDonald, C.E. and Chen, L.L. (1965) Anal. Biochem. 10, 175—177
11 Ferguson, A.R., Katsunuma, T., Betz, H. and Holzer, H. (1973) Eur. J. Biochem. 32, 444—450
12 Betz, H., Hinze, H. and Holzer, H. (1974) J. Biol. Chem. 249, 4515—4521
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Radall, R.J. (1951) J. Biol. Chem, 193, 265—275
14 Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaki, M., Hamada, M. and Takeuchi, T. (1970) J. Antibiot. 23, 259—262
15 Aoyagi, T., Kunimoto, S., Morishima, H., Takeuchi, T. and Umezawa, H. (1971) J. Antibiot. 24, 687—694
16 Ulane, R.E. and Cabib, E. (1974) J. Biol. Chem. 249, 3418—3422
17 Hayashi, R., Oka, Y. and Hata, T. (1969) Agric. Biol. Chem. 33, 196—206
18 Hayashi, R. and Hata, T. (1972) Agric. Biol. Chem. 36, 630—638
19 Matern, H., Hoffmann, M. and Holzer, H. (1974) Proc. Natl. Acad. Sci. U.S. 71, in the press
20 Saheki, T., Matsuda, Y. and Holzer, H. (1974) Eur. J. Biochem. 47, 325—332