COMPARTMENTATION OF INHIBITORS OF PROTEINASES A AND B AND CARBOXYPEPTIDASE Y IN YEAST

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SUMMARY: After centrifugal fractionation at 40,000 x g of a metabolic lysate from yeast spheroplasts proteinases A and B, and carboxypeptidase Y were found exclusively in the sediment, whereas inhibitors of these proteinases were present only in the supernatant. Immunoprecipitation with an antiserum prepared against the pure heat-stable proteinase B-inhibitor occured in the supernatant but not in the extract of the particulate fraction.

The purification and some properties of a macromolecular, heat-stable material inhibiting the tryptophan synthase inactivating enzymes I and II from yeast have been described by Ferguson et al. (1). The identity of the tryptophan synthase inactivating enzymes I and II with the earlier described yeast proteinases A as well as B, described by Saheki and Holzer (2), showed that Ferguson et al. actually purified proteinase inhibitors. By further fractionation of the heat-stable inhibitory material two specific inhibitors for proteinase B (3) and two other specific inhibitors for proteinase A (4) have been isolated. Hayashi et al. (5) have isolated a proteolytically inactive complex of carboxypeptidase Y (previously called proteinase C) with a molecular weight of 82,000. Partial denaturation or proteolysis of the inac-

tive complex leads to active carboxypeptidase Y with a molecular weight of 62,000 (6,7). The existence of a carboxypeptidase Y-inhibitor with a molecular weight of 20,000 has been discussed by Hayashi et al. (6,7) and has been reported by Lenney et al. (8,9).

It is demonstrated in the present paper that a soluble fraction containing carboxypeptidase Y inhibiting material is obtained by high-speed centrifugation of a metabolic lysate of yeast spheroplasts. Furthermore it is shown that the earlier described inhibitors of the proteinases A and B are also localized in the soluble fraction of the lysate. In contrast to the localization of the inhibitors the proteinases A and B and carboxypeptidase Y are present in the sedimentable fraction of the lysate which contains the vacuoles. This agrees with previous results of other authors (10,11,14,15).

MATERIALS AND METHODS.

Sources of chemicals and the method of protein determination were the same as described in previous papers (3,11). The assay of carboxypeptidase Y was performed as described by Trautschold et al. (12) with the following modifications: The reaction mixture at pH 8.0 contained 10 mM N-acetyl-L-tyrosine ethylester dissolved in dimethylsulfoxide (final concentration 1.3 %) as substrate. Carboxypeptidase Y activity was expressed as units, 1 unit defined as 1 µmol of N-acetyl-L-tyrosine ethylester hydrolyzed per min at 25°. In samples containing ethanol, carboxypeptidase Y activity was determined after filtration on a Sephadex G-25 column (0.5 x 3 cm). Proteinases A and B were assayed as described by Saheki et al. (2). The methods of culturing the

diploid yeast Saccharomyces cerevisiae X 2180 (aα) on YEPD medium at 30° was carried out as described by Hasilik et al. (11). Cells were harvested after a growth period of 20 hrs, and spheroplasts and metabolic lysate prepared by a modification of the procedure of Indge (13) as reported by Hasilik et al. (11). 1 ml lysate corresponded to 0.3 g of yeast (wet weight). Fractionation of the metabolic lysate was performed by centrifugation at 40,000 x g for 20 min. The sediment obtained was washed with 0.9 M imidazole chloride buffer, pH 6.4, containing 7 % mannitol (w/v), suspended in the same buffer and diluted to the original volume. For the determination of enzyme activities in recombined sediment and supernatant (Table I) the fractions were mixed in a ratio corresponding to the composition of the original lysate. All samples containing lysate and 40,000 x g sediment were sonicated before the enzyme activities were determined as described by Hasilik et al. (11). The preparation of a specific antiserum against the yeast proteinase B-inhibitors in rabbits has been reported earlier (3). The extraction of proteinase B-inhibitors from cell fractions for the purpose of immunoassay was carried out by heating at 95° for 20 min, followed by centrifugation for 4 min at 8000 x g.

RESULTS AND DISCUSSION.

A metabolic lysate containing intact vacuoles was fractionated by centrifugation at 40,000 x g in a supernatant and a sedimenting fraction. The proteolytic activities as measured with specific assays for proteinases A and B and carboxypeptidase Y, respectively, in the total ultrasonicated lysate, the supernatant, the sediment, and the recombined supernatant plus sediment are

Table I

Proteinase activities in fractions of a metabolic lysate from yeast spheroplasts

Exp.No	Preparation	Units/ml lysate		
		Proteinase A	Proteinase B	Carboxypeptidase Y
1	Lysate	16.7	< 0.001	0.182
2	Supernatant	1.9	< 0.001	0.009
3	Sediment	38.4	0.148	1.05
4	Supernatant plus Sediment	20.4	< 0.001	0.123

The metabolic lysate was fractionated by centrifugation at 40,000 x g for 20 min at 0^{o}.

shown in Table I. In accordance with earlier observations (10, 11,14,15), the bulk of the proteolytic activity was present in the sediment, which contains the yeast vacuoles. Only traces of proteolytic activity are found in the supernatant. In experiments not shown in Table I, it was demonstrated that centrifugation at 100,000 x g leads to the same results as centrifugation at 40,000 x g. Combination of supernatant and sediment in proportions present in the original lysate yielded nearly the same proteinase activities as observed in the lysate (see Table 1, experiment No.4). Under the conditions used in our experiments proteinase B is completely inhibited, whereas carboxypeptidase Y is inhibited to about 85 % and proteinase A to about 50 %. It was demonstrated in experiments not shown in Table I, that after boiling of the supernatant fraction, the inhibitory activity of this fraction remained unchanged for proteinases A and B, whereas for carboxy-

peptidase Y the inhibitory activity disappeared completely. This differential temperature sensitivity of the inhibitors is in agreement with earlier findings (1,3,4,9,16).

The inhibitors for proteinase A (4) and carboxypeptidase Y (17) are at present available only in very small amounts. Therefore, only in the case of proteinase B-inhibitor we were able to demonstrate the cellular compartmentation of this inhibitor by specific immunoprecipitation. It has been described earlier (3) that a specific antiserum against both proteinase B-inhibitors is easily obtained by immunisation of rabbits with purified proteinase B-inhibitor. Immunoprecipitation of boiled samples of the fractions used in the experiments summarized in Table I with this antiserum is shown in Fig.1. A detectable amount of immunoprecipitate is formed only with boiled lysate or boiled

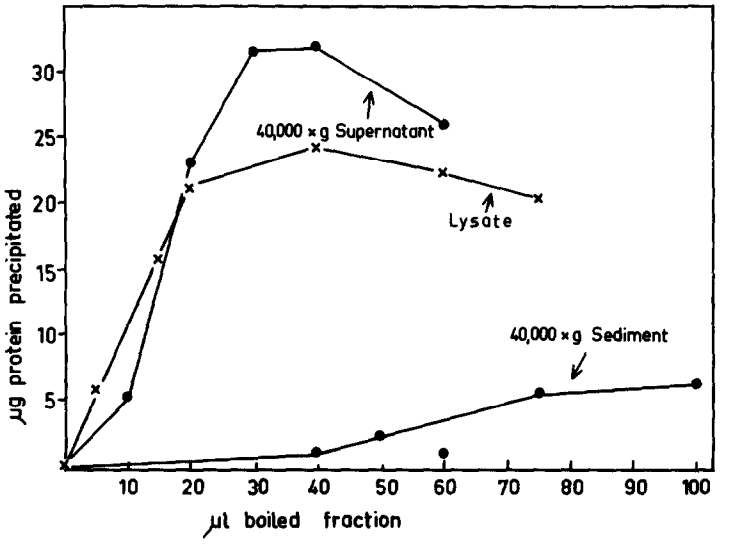

FIGURE 1. Immunoprecipitation of yeast proteinase B-inhibitor from fractions of a "metabolic lysate" from yeast spheroplasts. The boiled fractions prepared as described in "Materials and Methods" were used as sources of proteinase B-inhibitor. Varying amounts of these boiled fractions were precipitated overnight with 50 µl antiserum in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 M NaCl and 0.02 % sodium azide in a total volume of 0.16 ml at room temperature. After centrifugation and repeated washing the protein content of the precipitates was determined.

Table II

Proteinase B-inhibitor in boiled fractions from metabolically lysed spheroplasts from Saccharomyces cerevisiae X 2180

Fraction	µg Proteinase B-inhibitor/ml lysate
Lysate	17.9
40,000 x g Supernatant	16.1
40,000 x g Sediment	0.96

The inhibitor content was estimated by quantiative immunoprecipitation as described in the legend to Fig.1. Since it has been shown that S.cerevisiae X 2180 contains almost exlusively proteinase B-inhibitor II (as will be described in detail later by Betz et al.), a calibration curve obtained with pure proteinase B-inhibitor II isolated as described earlier (3) was used for the calculation of the data.

40,000 x g supernatant, but not with corresponding amounts of boiled extract of 40,000 x g sediment. A quantitative evaluation of the immunological analysis (Fig.1) is shown in Table II. For immunological calibration pure proteinase B-inhibitor (3) was used. It may be seen that only small amounts (about 5 %) of the inhibitor are present in the sediment fraction whereas about 95 % are found in the supernatant.

The different compartmentation of proteinases and proteinase inhibitors in yeast provides interesting possibilities for the mechanisms of control of specific proteinase action. Some possibilities are discussed in recent reviews from this laboratory (18,19).

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