CARBOXYPEPTIDASE S FROM YEAST: REGULATION OF ITS ACTIVITY DURING VEGETATIVE GROWTH AND DIFFERENTIATION

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1. Introduction

To uncover the physiological role of the so far known intracellular proteinases of the yeast *Saccharomyces cerevisiae* – proteinase A, proteinase B, carboxypeptidase Y and three aminopeptidases (reviewed [1,2]) – we had started a genetic and biochemical approach trying to isolate mutants of these enzymes and to study the link between proteinase deficiency and the biological event catalyzed by this enzyme [3]. The first attempts had led to the isolation of a carboxypeptidase Y mutant [3]. Biochemical analysis of this mutant had uncovered the existence of a second enzyme exhibiting carboxypeptidase activity, which we called carboxypeptidase S [4,5]. In contrast to carboxypeptidase Y, which is a serine peptidase [1,2] the activity of the new carboxypeptidase S was shown to be metal dependent [4]. To learn more about the function of this new enzyme, we report here studies on its regulation during vegetative growth and the differentiation process of sporulation.

2. Materials and methods

L-Amino acid oxidase type I, horseradish peroxidase and *O*-dianisidine dihydrochloride were purchased from Sigma (München). *N*-Cbz–Gly–L-Leu was obtained from Bachem (Bubendorf). All yeast media were from Difco. All other chemicals used were obtained from Merck (Darmstadt).

The carboxypeptidase Y-less *Saccharomyces cerevisiae* strain 4545/III, 2–1 A (α, prc 1–1) [3] was used in all studies following carboxypeptidase S-activity. For sporulation studies diploids were constructed harboring the same genotype. For measuring proteinase B and carboxypeptidase Y, wild type strain S 288 c (α, mal$^-$) [3] was used. To test the influence of carbon source on carboxypeptidase S activity cells were grown at 30°C in 0.67% yeast nitrogen base without amino acids and 2% glucose or 2% ethanol added. For test of influence of nitrogen source, cells were grown on 0.17% yeast nitrogen base without amino acids and ammoniumsulfate, 2% glucose and the desired nitrogen source added. All media were adjusted to pH 6.8–7.0. Cell density was measured at 546 nm in an Eppendorf spectrophotometer. For sporulation studies, cells were grown 24 h at 30°C on complete medium, centrifuged, washed twice and transferred into 1% potassium acetate sporulation medium such that cell density ranged from $1–2 \times 10^7$ cells/ml. Crude extracts from vegetatively grown cells were prepared by suspending cells in 0.15 M imidazole chloride buffer, pH 7.5 (1:1, w/v) and breaking them in a French pressure cell (Aminco, Silver Spring, MD). Sporulated cultures were broken by sonifying cells suspended in buffer (1:2, w/v) and mixed with glass beads (0.45–0.5 mm diameter) 2 g/ml cell suspension 4×30 s at 10 A with cooling intervals of 30 s (Branson, Offenbach). Carboxypeptidase S was assayed as in [4]. As free amino acids interfere with the assay, crude extracts were dialyzed 2 times against 0.1 M imidazole buffer and 2×10^{-4} M $ZnSO_4$ added. As carboxypeptidase S activity can be activated at room temperature at acidic pH, dialysis was carried out for 20–36 h at pH 5.3 and 25°C (see section 3). Carboxypeptidase Y

was measured as in [6] and proteinase B was assayed as in [7]. Protein was determined according to [8] using bovine serum albumin as standard.

3. Results and discussion

It had been shown, that activities of proteinase A, proteinase B and carboxypeptidase Y in crude extracts were only measurable after incubation of the extracts at around pH 5 for several hours at 25°C [7,9]. This treatment leads to activation of proteinase activities due to autocatalytic cross inactivation of their respective inhibitor proteins [1,2] triggered by proteinase A, which partly dissociates from its inhibitor under those conditions [7]. Table 1 shows, that also carboxypeptidase S can be activated more than 2-fold by treatment of the crude extract, at pH 5.3. Thus all measurements of carboxypeptidase S levels were done after this activation procedure. Whether inhibition of carboxypeptidase S in fresh crude extracts is due to a specific inhibitory protein or due to unspecific binding of polypeptides, is under investigation.

Figure 1 shows carboxypeptidase S levels during different growth phases in medium containing glucose as carbon source. As can be seen, carboxypeptidase S activity shows a slight increase of less than 2-fold, when cells leave logarithmic growth phase and enter the beginning of diauxic phase. This increase levels off again in stationary phase. Proteinase A, proteinase B and carboxypeptidase Y activities had been shown to be repressed by glucose and derepressed in stationary phase cells or grown on a non-fermentable carbon source [9,10]. Carboxypeptidase S activity behaves differently: There is no significant difference in activity in log phase cells when glucose is present, as compared to stationary phase when cells starve for glucose. In addition, growth of cells on the non-fermentable carbon source ethanol leads to a level of 9 units/mg, not very different from glucose grown cells (fig.1). One might suspect, that the slight increase of carboxypeptidase S activity at the begin-

Table 1
Carboxypeptidase S activity in non activated and activated crude extracts

Spec. act. (U/mg)	
Non-activated extract	Activated
2.8	6.4

Cells were grown 48 h in yeast nitrogen base medium. Non-activated extracts were dialyzed as in section 2, at pH 7.0 and 0–4°C. For activation, extract was incubated as in section 2

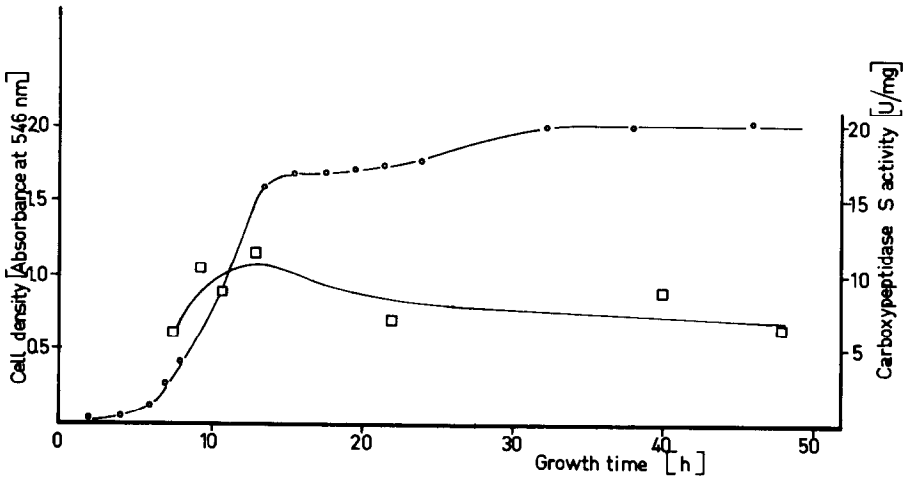

Fig.1. Growth of yeast strain 4545/III, 2–1 A (prc 1–1) and activity pattern of carboxypeptidase S. Growth proceeded in medium containing 2% glucose and 0.67% yeast nitrogen base without amino acids. Test was performed as in section 2. (○—○) Cell growth; (□—□), carboxypeptidase S activity.

Table 2
Carboxypeptidase S activity during cell growth on different nitrogen sources

Nitrogen source	Spec. act. (U/mg)
$(NH_4)_2SO_4$	6.4
Casaminoacids	6.2
Leucine	24.5
Valine	20.7
N-Cbz–Gly–Leu	80.0
N-Cbz–Gly–Leu + casaminoacids	7.0

Cells were grown in glucose as carbon source into early log phase. Additions were: $(NH_4)_2SO_4$, 0.5%; casaminoacids, 1.5%; leucine, 1%; valine, 1%; *N*-Cbz–Gly–Leu, 1%. Test was performed as in section 2

ning of the diauxic growth phase might, however weak, be a response necessary for the cells' adaptation of the protein pattern from fermentative to respirative growth which is supposed to be accompanied by protein breakdown of now unnecessary proteins [11]. The influence of nitrogen source on carboxypeptidase S levels is shown in table 2: growth on a mixture of amino acids (casaminoacids) leads to carboxypeptidase S activity similar to that found for growth on ammonium (fig.1). However growth on a single amino acid, leucine or valine, leads to about 3–4-fold increased carboxypeptidase S activity compared to growth on ammonium or a mixture of amino acids. A striking increase of carboxypeptidase S activity is visible when cells are grown on the dipeptide *N*-Cbz–Gly–Leu as sole nitrogen source (table 2): carboxypeptidase S level rises 10–12-fold over level found for growth on ammonium or an amino acid mixture. As can be seen from table 2 this drastic increase is absolutely repressed in the presence of casaminoacids. This finding led to the investigation of activity levels of the endoproteinase B and of carboxypeptidase Y after growth on the dipeptide. Whereas activity of proteinase B is not altered (not shown here), activity of carboxypeptidase Y was found to be 0.15 units/mg under these growth conditions as compared to 0.025 units/mg when grown on ammonium [3]. Thus, growth on the dipeptide *N*-Cbz–Gly–Leu leads also to a drastic increase of 6-fold for carboxypeptidase Y activity.

The activity level of carboxypeptidase S during sporulation was also investigated. Since the differentiation event of sporulation in yeast occurs in the absence of exogenous nitrogen source, the internal amino acid pool and the supply of nitrogen compounds from protein turnover must be sufficient for new protein synthesis [12]. This points to the action of proteinases during this event and the question of a sporulation specific increase of proteinases arises. A several-fold increase of proteinase A and B activities had been found in sporulating cells during the first 20–24 h of sporulation [13,14]. There is disagreement, however, about a sporulation-specific increase of carboxypeptidase Y activity [13,14]. Carboxypeptidase S activity was followed 24 h in a diploid strain, which is able to sporulate, until the first asci appeared and, for comparison, in a haploid strain, not able to sporulate. Measurement of the enzyme during different times of cells in sporulation medium showed an average value of around 16 units/mg throughout in diploid and haploid cells. Thus, even though there is a slight increase of carboxypeptidase S under absolute nitrogen starvation in haploid and diploid cells, there is no sporulation specific enhancement of the enzyme.

Taken together, these studies show that carboxypeptidase S, unlike proteinase A and B and carboxypeptidase Y, is very little, if at all, regulated by the carbon source. In contrast, nitrogen source seems to play a prominent role: there is a moderate increase of activity by growth on poor nitrogen source like the amino acids leucine or valine. A drastic increase of carboxypeptidase S activity, probably due to induction, is brought about by cell growth on the dipeptide *N*-Cbz–Gly–Leu. This increase can be repressed by casamino acids. A similar increase of activity by the dipeptide could be shown for carboxypeptidase Y. This points to a prominent role of these two carboxypeptidases in supplying the cell with amino acids required for growth from extracellularly supplied peptides when ammonium or amino acids are not available. As could be shown [15], in contrast to wild type, a mutant lacking both carboxypeptidases is unable to grow when *N*-Cbz–Gly–Leu is supplied as sole nitrogen source. The rather constant but considerable amount of carboxypeptidase S activity during all phases when grown on glucose and ammonium and during differentiation probably

reflects an additional function of this enzyme which is common to all growth phases and sporulation. The isolated mutants lacking carboxypeptidase S activity [15] will most probably give an answer.

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