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# PARTIAL CHARACTERIZATION OF INTRACELLULAR PROTEASE ACTIVITY WHICH PARTICIPATES IN THE INACTIVATION OF CARBAMYL PHOSPHATE SYNTHASE IN *PHYCOMYCES BLAKESLEEANUS*

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We have partially characterized an intracellular fraction from *Phycomyces blakesleeanus* which shows proteolytic activity. The apparent thermal inactivation constant (Kd) was $0.12\,min^{-1}$ at 50°C. This proteolytic fraction was split into two active fractions by ultrafiltration using a membrane with an exclusion size of 30,000. Both fractions were inhibited by phenyl methyl sulphonyl fluoride. The $K_i$ value for the fraction with molecular weight > 30,000 was 0.075 mM. The fraction with molecular weight < 30,000 inactivated the *Phycomyces* CPS.

KEY WORDS: Intracellular protease activity, carbamyl phosphate synthase, CPS, *Phycomyces*

## INTRODUCTION

Limited proteolysis has been identified as a mechanism that regulates many physiological enzyme activities. *Phycomyces* is a unicellular zygomycete fungus which shows multiple responses to different stimuli.[1,2] Fischer and Thomson[3] pointed out the existence of three serine proteinases of *Phycomyces blakesleeanus* which were found to enhance chitin synthase activity in *Phycomyces*. It has been suggested that activation of chitin synthase is part of the growth responses of the *Phycomyces* sporangiophore.[4] Carbamyl phosphate synthase (CPS) from *Phycomyces* has been purified by us and some of its properties are described here. The *Phycomyces* CPS uses ammonia as a primary N-donor and requires free $Mg^{2+}$ for maximal activity but does not require activation by N-acetyl-L-glutamate and does not use L-glutamine as a nitrogen donating source. The purified form of the enzyme was found to be very unstable although the enzymatic preparations during the purification procedure were found to be very stable regarding CPS activity.

It has been reported that the bifunctional enzyme CPS/aspartate carbamyl transferase of *Neurospora crassa* is the target of an intracellular protease.[5] These authors suggest an instability in the tertiary structure of the enzyme. Proteolytically induced changes have also been shown to occur in the molecular form of the CPS-uracil-aspartate *trans*-carbamylase complex coded for by the URA2 locus in *Saccharomyces cerevisiae*.[6]

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Paulus and Switzer[7] reported that CPS isozymes of *Bacillus subtilis* were inactivated at the end of the exponential growth period. It has been shown that the inner mitochondrial membrane participates in the inactivation and possibly the initial degradation of CPS from rat liver at neutral pH.[8] CPS (glutamine-dependent) activity could not be demonstrated in crude extracts from *Toxoplasma gondii*[9] due to the presence of an inhibitory component.

This paper reports a study of protease activity in *Phycomyces* related to the instability of CPS.

## MATERIALS AND METHODS

### *Biochemical reagents*

Dithiothreitol (DTT), NADH, $KHCO_3$, glycylglycine, magnesium acetate and thiamine hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, USA. ATP, lactate dehydrogenase (LDH), pyruvate kinase (PK), phosphoenolpyruvate (PEP), pepstatin, leupeptin, $\alpha_1$-antitrypsin and phenyl methyl sulfonylfluoride (PMSF) were from Boehringer Mannheim, Mannheim, West Germany. Bacto-casitone, Bacto-Agar and skimmed milk were purchased from Difco, Detroit, MI, USA. Reagents for polyacrylamide electrophoresis (PAGE) were from Shandon Southern Products Ltd, Cheshire, UK. Triton X-100 and all other reagents were of standard analytical grade and provided by Merck, Darmstadt, West Germany.

### *Media and growth conditions*

The wild type NRRL 1555(−) *P. blakesleeanus* strain was grown in a liquid minimal medium as described by Sutter.[10] *Phycomyces* spores were heat-shocked by incubating an aqueous suspension containing $10^9$ spores/ml for 15 min at 48°C. Erlenmeyer flasks (250 ml capacity) containing 100 ml of liquid minimal medium were inoculated with $10^6$ spores/ml of the spore suspension. The culture was incubated in a New Brunswick "Psychroterm G-27" rotary shaker at 20°C and 200 rpm. Mycelia of *Phycomyces* were obtained by filtration after 36–48 h of growth for CPS activity and 72 h for proteolytical activity.

### *Mycelial dry weight determination*

Growth of *P. blakesleeanus* was determined as a function of the mycelial dry weight. Aliquots of the culture were passed through a GF/C Whatman glass fibre filter. They were heated to constant weight at 80°C.

### *Protein determination*

Protein was determined by the Biuret method[11] with bovine serum albumin as a standard.

### *Cell-free extracts*

The mycelium, harvested by filtration, was washed thrice with distilled water and then cut into pieces and suspended in 50 mM triethanolamine buffer, pH 7.8, containing

15 mM magnesium acetate and 1 mM DTT at a ratio of 1 ml to gram of weight. The suspended mycelium was homogenised in a Braun MSK homogenizer cell-disruptor for 45 sec. The homogenate was centrifuged at 16,000 g for 25 min at 4°C and the pellet discarded. The supernatant solution was used for assay of proteolytic activity.

*Carbamyl phosphate synthase assay*

CPS activity was determined according to the method of Guthörlein and Knappe[12] as modified by Chabas *et al.*[13] using a coupled enzymatic assay with pyruvate kinase and lactate dehydrogenase for measurement of NADH oxidation.

In a final volume of 1.0 ml the assay mixture contained $KHCO_3$ (50 mM), $(NH_4)_2SO_4$ (35 mM), ATP (5 mM), PK (14 U), PEP (2.5 mM), LDH (4.5 U), $MgSO_4$ $7H_2O$ (15 mM), 2-mercaptoethanol (MSH) (5 mM) NADH (0.5 mM) and 200 $\mu$l of the enzymatic preparations suitably diluted in 50 mM glycylglycine buffer, pH 7.4. The blank contained all reagents as in the assay mixture except NADH and the enzymatic preparation. Activity was measured at 30°C by the decrease in $A_{340}$ associated with NADH oxidation in a Beckman model 25 spectrophotometer equipped with a recorder and temperature control unit. Under these conditions the molar absorption coefficient for NADH was $5.186 \times 10^3\ M^{-1}1^{-1}$. One unit of CPS is defined as the amount of enzyme that catalyzes the synthesis of 1 $\mu$mol of carbamyl phosphate (NADH) per minute at 30°C.

*Protease activity assay*

Protease activity was estimated by the measurement of the extent of hydrolysis of the substrate casein by the hole plate method. The reaction was performed in a 1 cm diameter hole made on a 90 mm diameter Petri dish containing 30 ml of solid supporting medium which contained 0.5% skimmed milk and 20% Bacto-agar in distilled water. The assay mixture contained casein supplemented with 10 $\mu$l of antibiotic suspension as described by Hansen *et al.*[14] together with the samples to be assayed (150–200 $\mu$l). After standing for 1 h at 4°C the dishes were incubated for 48 h at 38°C and afterwards the extent of reaction was measured by the lysis produced in the hydrolyzed zone. These values were taken as being proportional to the protease activity of the samples[15] and were linearly related to the amount of the samples used (100–200 $\mu$l).

*Polyacrylamide-gel electrophoresis*

Polyacrylamide-gel electrophoresis was used as a means of evaluating the purity of the protease activity. It was performed in 7.5% acrylamide gels, pH 8.3 by a technique similar to that of Davis.[16] After electrophoresis the gels were stained with Coomassie Brilliant Blue.

## RESULTS AND DISCUSSION

We have determined the time course of protease activity during the growth of *Phycomyces*. The activity was measured both in the culture medium for extracellular proteases and in the cell-free extract for intracellular proteases. To the homogenization buffer we have added, where indicated, 0.1–1% Triton X-100. Extracellular

protease activity was not detected even when the culture medium was 50-fold concentrated. As can be seen (Figure 1) the time course for intracellular protease activity paralleled the growth curve of *Phycomyces*. The specific activity reached its maximum value after three days of growth and increased with the presence of 0.1% Triton X-100 in the homogenization buffer. This effect was not enhanced by increasing the concentration of Triton X-100 to 1%. So the three-day-old mycelium of *Phycomyces* was used as the source of protease activity in further experiments. The supernatant obtained from this source, as described in the Materials and Methods section, showed protease activity as well as CPS activity.

For separation purposes only, this fraction was subjected to thermal inactivation experiments. The results shown in Figure 2 indicate that the intracellular protease activity was quickly inactivated at temperatures above 45°C. From a plot of log of the percentage of residual activity vs time from the data shown in Figure 2 a value of 0.129 $min^{-1}$ was calculated for the apparent inactivation constant of protease activity at 50°C. Since the CPS activity was also inactivated in the same temperature range, an increase in the temperature as a means of separating protease and CPS activity could not be used. So, the original fraction before heating was passed through a Diaflo PM 30 ultrafiltration membrane under 2 atm of $N_2$ pressure. Ninety percent of the protease activity was retained on the membrane. This fraction with molecular weight over 30,000 also contained all the CPS activity. In contrast, the filtered fraction, with molecular weight under 30,000 was devoid of CPS activity and when

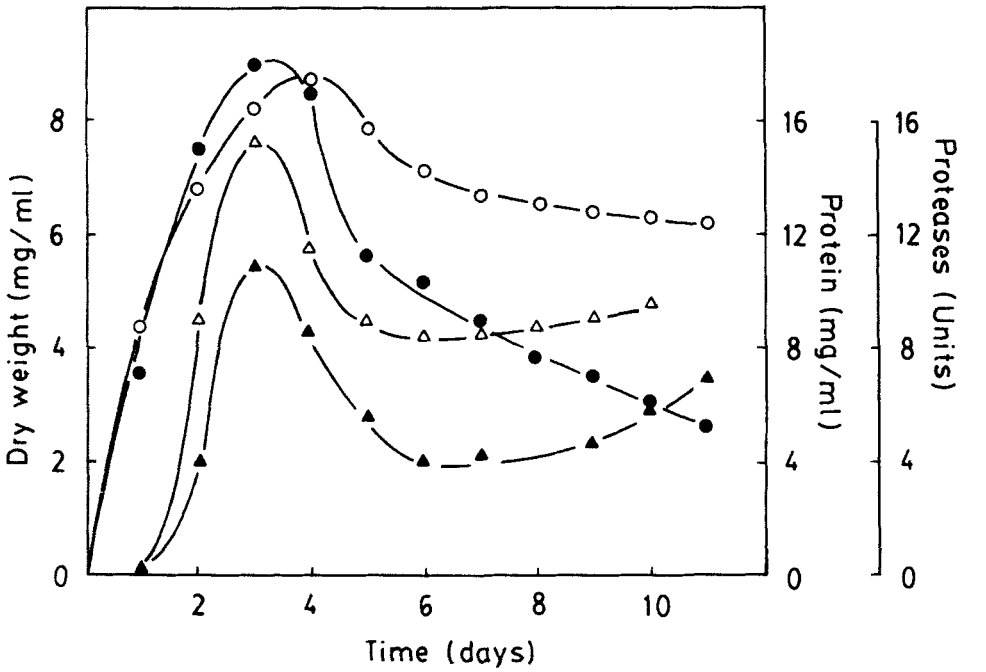


FIGURE 1 Time-course of *P. blakesleeanus* intracellular protease activity. *Phycomyces* was grown as described in the text and homogenized with (△) and without (▲) 0.1% Triton X-100. One unit of protease activity is defined as the amount of enzyme that produces a 1 mm depth of casein hydrolysis. *Phycomyces* growth (○) and the protein content (●) were measured as described in the text.

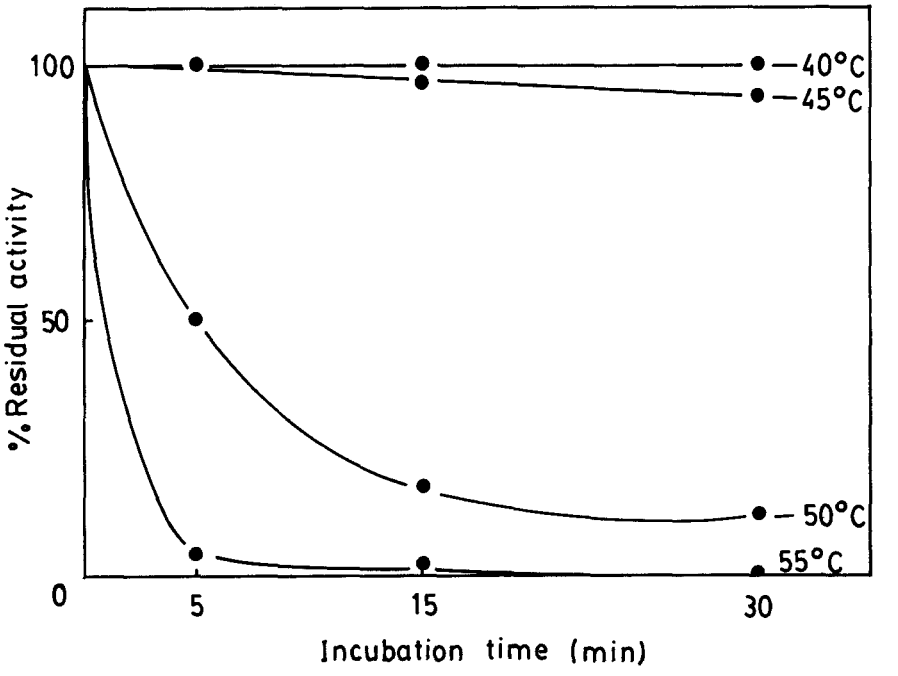


FIGURE 2 Effect of temperature on intracellular protease activity of *Phycomyces*. The experiments were carried out as described in the text. Aliquots of 200 $\mu$l were taken at the indicated time and assayed for protease activity.

concentrated (15-fold) in a rotaevaporator, showed some protease activity. These two protease fractions were subjected to thermal inactivation experiments; both showed a pattern which was similar to that obtained with the original fraction. From these experiments it may be concluded that the casein hydrolysis was produced by protease(s) from both fractions.

Protease activity was studied at pH values ranging between pH 7 and 8, where only type B protease activity is observed. Several protease inhibitors such as pepstatin, EDTA, phenylmethylsulfonyl fluoride (PMSF), leupeptin and antitrypsin were used as a means of characterising the proteolytic fractions. Both fractions were inhibited only by PMSF, which may indicate that they are composed mainly of serine proteases. As can be seen in Figure 3, the proteolytic fraction of molecular weight above 30,000 was inhibited by phenyl methyl sulfonyl fluoride with a calculated $K_i$ value of 0.075 mM. The fraction with molecular weight $< 30{,}000$ gave the same pattern (data not shown here).

Our aim here was to confirm that *Phycomyces* CPS could be inactivated and/or proteolytically degraded by neutral proteases such as those described for rat liver carbamyl phosphate synthase.[8] Experiments were conducted to establish whether intracellular neutral protease activity recognized the *Phycomyces* CPS as substrate at pH 7.4 which is the pH value of maximum activity of the purified enzyme (data to be published elsewhere). The protease fraction with molecular weight $< 30{,}000$ was exclusively used since CPS activity was absent in this fraction whereas the protease fraction with molecular weight $> 30{,}000$ contained all the CPS activity from the

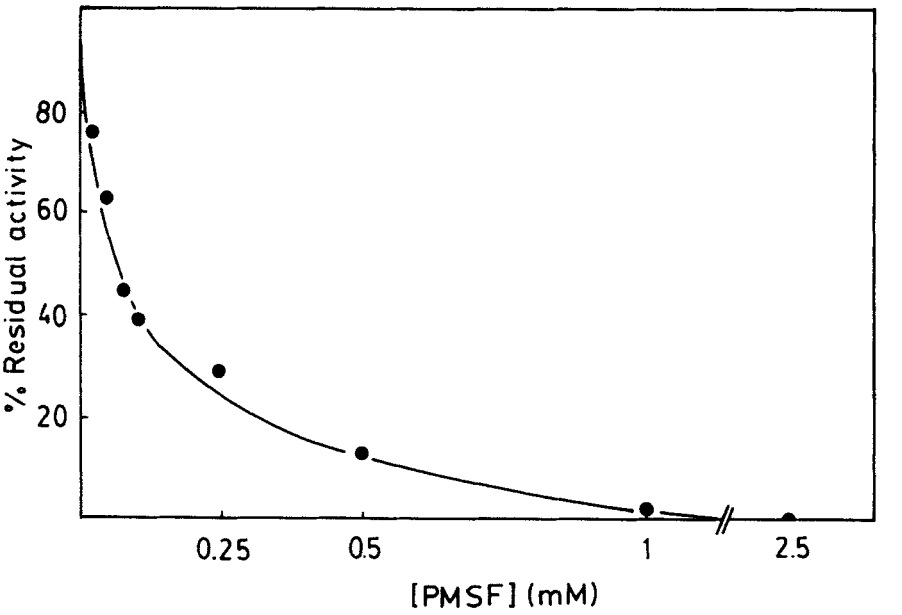


FIGURE 3 Inhibition by PMSF of *Phycomyces* protease activity. The molecular weight protease fraction over 30,000 was assayed for casein hydrolysis in the presence of the indicated concentrations of PMSF without prior incubation.

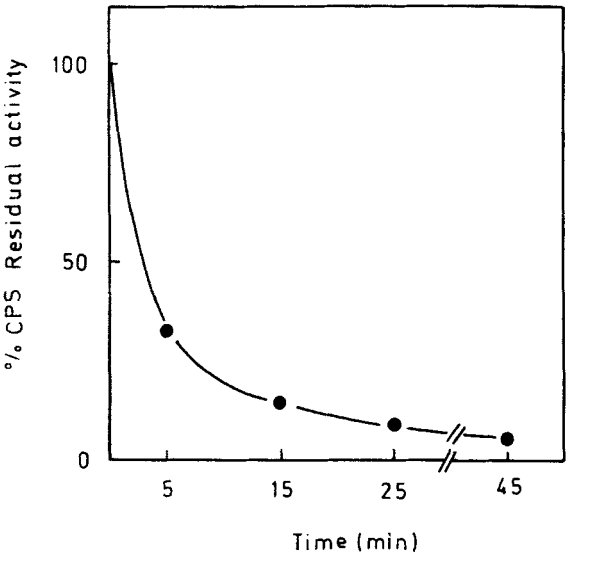


FIGURE 4 Inactivation of *Phycomyces* CPS by incubation with an intracellular protease at 30°C. In a set of experiments, four tubes in duplicate containing purified CPS (200 $\mu$l) and the protease fraction with molecular weight under 30,000 (200 $\mu$l) were incubated at the indicated time. Aliquots (200 $\mu$l) of each tube was tested for CPS activity.

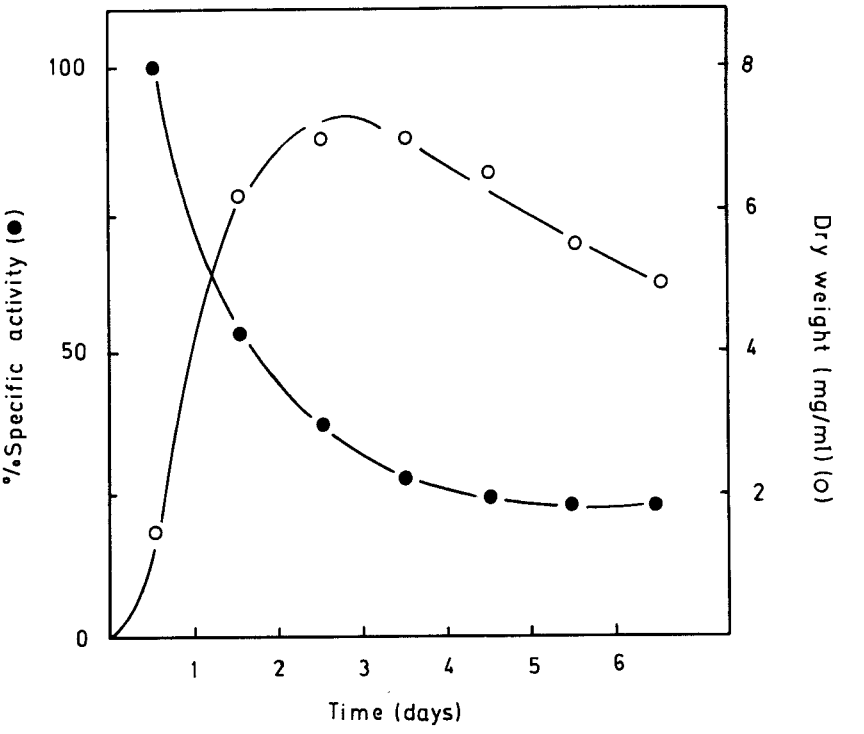


FIGURE 5 Time-course of *Phycomyces* CPS. Dry weight and CPS activity were determined as described in Materials and Methods section.

original fraction. In addition, CPS activity from the latter fraction was found to be very stable when stored at room temperature for a period of up to 12 h.

When the protease fraction of molecular weight < 30,000 was incubated at neutral pH, both at 0°C and 30°C, it inactivated the purified form of CPS from *Phycomyces*. The *Phycomyces* CPS lost almost all of its enzyme activity between 5–15 min (Figure 4). When this proteolytic fraction was subjected to PAGE at 7.5%, a major protein band was obtained with an $R_f$ value of 0.62 which could correspond to the protease(s), which recognizes the *Phycomyces* CPS as a substrate.

The co-purification of these type B proteases and the CPS activity may be indicative that this protease system may act on the CPS "*in vivo*" at the end of the exponential growth, coincident with the maximal protease production (Figure 5), in agreement with the observations described by Paulus and Switzer[7] for *Bacillus subtilis* OTCase. In addition, Rigby and Radford[5] reported that the loss of CPS activity and the change in the molecular weight from *Neurospora* appeared to be due to proteolytic activities, which may act in sequential order.

*Acknowledgements*

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