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PROTEASE AND CAROTENOGENESIS IN *BLAKESLEA TRISPORA*

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Key Word Index—*Blakeslea trispora*; Mucorales; carotene; trisporic acid; protease; inhibitor proteins.

Abstract—Carotene production by single and mated *Blakeslea trispora* has been studied. On mating and on the addition of trisporic acid to minus cultures there was an increase in the membrane bound neutral protease (MW 126 000) activity. The protease probably acts by inactivating the inhibitory protein of carotene biosynthesis resulting in increased carotenogenesis.

INTRODUCTION

The production of β-carotene by heterothallic *Blakeslea trispora* has received considerable interest in recent years. Though single cultures of this mould are carotenogenic, mating produces a 10–15-fold increase in carotene production [1]. This is attributed to the production of a sex hormone, trisporic acid, which derepresses some key enzyme(s) in the carotene biosynthetic pathway [2, 3]. A protein inhibitor of carotene biosynthesis has been isolated from single cultures [4]. This inhibitor disappears on mating and other proteins appear and as a result carotene formation is stimulated [4]. The present investigation elucidates the role of proteases in the regulation of inhibitory protein activity and hence carotenogenesis in *B. trispora*.

RESULTS AND DISCUSSION

It has been observed earlier that mating of single cultures, and the addition of trisporic acid to single cultures of *B. trispora* result in an increase in proteolytic activity [5]. Investigations were carried out therefore to study the role of proteases in the regulation of carotenogenesis in *B. trispora*. Soluble and particulate proteases from 72-hr-old minus cultures, minus cultures supplemented with optimum concentration of trisporic acid [6, 7], and mated cultures were studied (Fig. 1). Particulate neutral proteases from mated and trisporic acid supplemented cultures showed a significant increase in specific activity as compared to minus cultures. Soluble and other particulate enzymes did not show any significant change indicating that the particulate neutral proteases may play an important role in the stimulation of carotene production on mating and on trisporic acid supplementation of single cultures.

Since earlier work in this laboratory has shown that carotenogenesis in *B. trispora* is regulated by inhibitory protein [4], the possibility existed that these proteases had a role to play in inactivating the inhibitor and thus increasing carotenogenesis. Consequently, the inhibitory proteins and the protease activities were studied at various time intervals of mating (Fig. 2). For the first 2 hr of mating there was no change in the protease and the inhibitor protein activity. However, after this period, there was a linear increase in the protease activity and a corresponding decrease in the inhibitor protein activity. The inhibitor protein activity was completely lost at the end of 6 hr. This showed the involvement of these proteases in mating stimulated carotenogenesis.

To study the interaction of the proteases with inhibitor protein the proteases were purified on Sephadex G-100 columns. The mating and trisporic acid stimulated proteases appeared in the fifth to seventh fractions (Fig. 3). The MW as determined by gel filtration is found to be 126 000.

The effect of various protease inhibitors on the purified protease activity was studied (Table 1). *p*-Chloromercuribenzoate (PCMB) and EDTA had no effect on the purified enzyme activity, suggesting that —SH groups

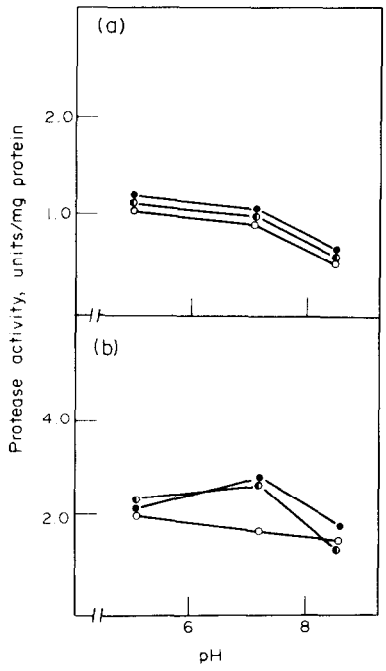

Fig. 1. Soluble (a) and particulate (b) protease activities from minus (○—○), minus supplemented with trisporic acid (◑—◑) and mated (●—●) cultures of *Blakeslea trispora*.

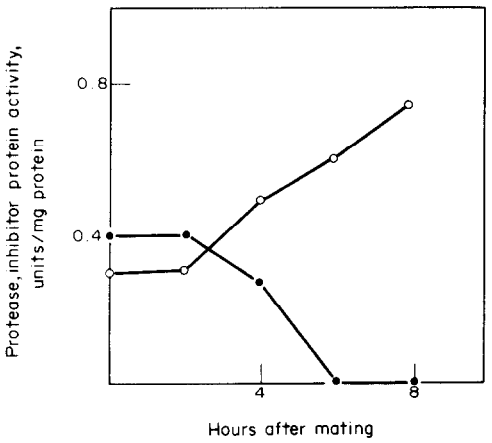

Fig. 2. Inhibitor protein (●—●) and membrane-bound protease (○—○) activities at different time intervals of mating of individual cultures of *Blakeslea trispora*.

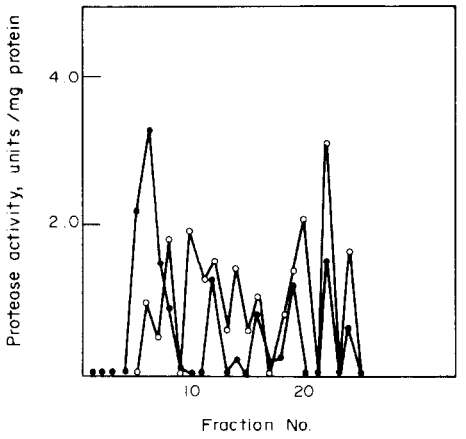

Fig. 3. Purification profile on Sephadex G-100 column of membrane-bound neutral proteases from minus (○—○) and mated (●—●) cultures of *Blakeslea trispora*.

Table 1. Effect of protease inhibitors on membrane-bound neutral protease activity from minus cultures, minus cultures with trisporic acid and mated cultures of *B. trispora*

Culture	Protease (Units/mg protein) Control	PMSF (1 mM)	PCMB (2 mM)	EDTA (5 mM)
Minus	1.96	0.35	2.1	2.22
Minus with trisporic acid	7.43	0.34	7.43	6.95
Mated	7.07	0.271	7.1	7.12

Table 2. Effect of *in vitro* incubation* of inhibitor protein with protease on carotenogenesis by *B. trispora* (−)

Incubation	Addition	Carotene (μg/g dry wt)	% Stimulation
1	None	439.08	—
2	1 + IP†	236.72	—
3	1 + minus protease	437.00	—
4	1 + TA‡ protease	438.00	
5	1 + mated protease	430.34	—
6	2 + minus protease	237.00	—
7	2 + TA protease	512.00	16.69
8	2 + mated protease	548.00	24.80

*1 mg of the inhibitor protein preparation was incubated with 1 mg of the protease before addition into the culture.
†IP: inhibitor protein.
‡TA: trisporic acid.

and metal ions were not involved with the activity of the protease. Phenylmethane sulfonylfluoride (PMSF) showed a marked inhibition in the activity of both the proteases suggesting the involvement of a serine group at the active site of the enzymes.

To test the effect of the proteases on the biological activity of inhibitor protein *in vitro*, the purified proteases were incubated with the partially purified inhibitor protein preparation for 1 hr at 37° and the inhibitor protein activity was measured (Table 2). It was observed that addition of inhibitor protein to the test system inhibited carotenogenesis. Proteases alone had no effect on carotene formation. There was no loss of inhibitor protein activity when it was incubated with normal protease from minus cultures. Incubation of inhibitor protein with mated and trisporic acid treated proteases resulted in a loss of inhibitor protein activity. There was not only an uplifting in the inhibitory activity of inhibitor protein but the protease incubation resulted in a significant enhancement in the carotene production as compared to the basal levels. Earlier reports have shown the presence of proteins stimulatory to carotene production [4]. Our data suggest that a modification of inhibitor protein may be stimulatory to carotene production.

Trisporic acid synthesis in *B. trispora* is initiated by the mating of single cultures [1]. As mating and the trisporic acid effect are very similar to each other, it is possible that on mating the production of trisporic acid results in a stimulation in the membrane-bound neutral serine protease. The protease probably acts on the carotene pathway by inactivating inhibitor protein.

EXPERIMENTAL

Mould strains and maintenance. The plus (+) (NRRL 2895) and the minus (−) (NRRL 2896) strains of *B. trispora* (obtained from U.S. Dept of Agric., Peoria, IL) were routinely maintained as described in ref. [8].

Medium for cultivation. The mould was grown on synthetic mucor medium (SMM) as described in ref. [9]. For expts with mated cultures, single cultures were grown for 48 hr in 250 ml flasks containing 50 ml medium. They were then mixed with the medium and incubated for a further 24 hr. Expts with single

cultures were performed by growing the cultures in 250 ml flasks containing 100 ml medium for a total period of 72 hr. Addition of trisporic acid was carried out after 48 hr of growth. Inoculum preparation, cultivation and growth measurement were done as described in ref. [10].

Preparation of cell-free extract. The frozen mycelia were crushed in a clean, unglazed, precooled mortar and pestle, an extract (10 w/v) was prepared in 0.1 M Tris–HCl buffer (pH 7.2) and centrifuged at 1000 g for 30 min. The supernatant was used for soluble enzyme assay. The pellet was washed 3 × with cold 0.1 M Tris–HCl buffer (pH 7.2), resuspended in the same buffer and treated with 0.1% Triton X 100 for 30 min and then centrifuged at 15 000 g for 30 min. The supernatant was used for the particulate enzyme assay. All operations were carried out between 0° and 5°.

Protein estimation. The protein was estimated by the method of ref. [11].

Protease assay. Protease assay was by the method of ref. [12]. One unit of the enzyme was defined as μmol tryptophan liberated/ml of enzyme per hr at 37°.

Gel filtration. Two columns of Sephadex G-100 (1.8 × 35 cm) were calibrated. The crude particulate protein from minus, minus supplemented with trisporic acid and mated cultures were loaded on to the columns; 5-ml fractions were collected and the protease activity was measured.

Isolation of inhibitor proteins. Inhibitor protein was isolated from 48 hr-old minus cultures. The frozen mycelia were crushed in a precooled mortar and pestle and an extract (10 w/v) was prepared with 0.1 M Tris–HCl buffer (pH 7.2). The extract was centrifuged at 15 000 g. The supernatant was brought to 0–60% satn with solid $(NH_4)_2SO_4$. The ppts were dissolved in the same buffer and dialysed overnight against cold distilled H_2O. The soln was used as the inhibitor protein preparation [4].

Biological activity of inhibitor protein. The inhibitor protein preparation after membrane filtration (0.45 μm pore size) was added aseptically to 48-hr-old minus cultures, incubated for a further 48 hr and the carotene was extrd and estimated. One unit of inhibitor protein was defined as the amount of protein which could cause 50% inhibition in carotene formation in the test system in 48 hr as described in ref. [4].

Extraction and estimation of carotenes. The carotenes were extrd in Me_2CO–Et_2O (1:1) and the total carotenoids were estimated as β-carotene as described in ref. [10].

Addition of inhibitors. From the purified fraction, the protease was assayed after the addition of phenylmethane sulfonylfluoride (1 mM), EDTA (5 mM) and PCMB (2 mM) to the assay mixture.

Addition of trisporic acid. Trisporic acid was extracted from mated *B. trispora* culture medium as described by Sutter [13]. The final $CHCl_3$ extract was evapd to dryness under red. pres. and the residue was dissolved in 0.1 M Tris–sulphate buffer (pH 7.2). The trisporic acid preparation was passed through a membrane filter (0.45 μm pore size) before addition into the 48-hr-old minus cultures.

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