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Behavior of phosphatase isoforms during sclerotium formation in *Physarum polycephalum*

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Abstract

The behavior of phosphatase isoforms under dark-starvation from plasmodium of *Physarum polycephalum* were investigated to determine their possible roles in sclerotium formation. Two and a half days after dark-starvation, approximately 95% of plasmodia plates formed sclerotia. Specific phosphatase activity increased markedly up to *ca.* two-fold within the first day of starvation, after which the enzymatic activity decreased rapidly to a level less than the initial level within 2 days of the starvation period. Among the two isoforms of enzyme detected just before sclerotization under dark-starvation conditions, the enzymatic activity of the major isoform (*Rm* value of 0.6) decreased gradually within 1.5 days of starvation, then linearly to less than 20% of that at the beginning of the observation. Those of other major isoform (*Rm* value of 0.7) increased up to *ca.* two-fold within the first day of starvation, then decreased linearly to levels less than that of the first 2 days of the starvation period. Behavior of this isoform strongly suggests that it initiates the formation of sclerotium under dark-starvation conditions.

Keywords: *Physarum polycephalum*; Myxomycetes; Slime mould; Plasmodia; Sclerotia; Dark-starvation; Phosphatase

1. Introduction

In previous papers we reported on a phosphatase [EC3.1.3.2] in a cytoplasmic soluble fraction of the plasmodium (macroplasmodium) of the true slime mould, *Physarum polycephalum* (Kaneko and Yamaura, 1982; Kaneko and Kato, 1990). Changes in relative activities of an acid phosphatase had also been reported by Hütterman et al. (1979) during differentiation (spherulation) of a microplasmodium, which was induced when a plasmodium was transferred to a non-nutrient salt medium (starvation). However, the biological function of the enzyme was unknown.

A plasmodium forms a macroplasmodium when grown on agar plates with sufficient nutrients, and forms a microplasmodium when grown in liquid culture. A plasmodium of the true slime mould *Physarum polycephalum*, a multinucleated cell, shows active shuttle streaming of the cytoplasm and rapidly grows under moist and nutrient conditions. When a plasmodium is deprived of nutrients and grown under dark-starvation conditions, it forms a cyst (sclerotium) as a non-growing dormant body (Guttes and Guttes, 1963). After shuttle streaming ceases, the plasmodium is surrounded by a thick wall (Jump, 1954), which protects it from environmental stresses. The transformation of microplasmodia into cysts occurs with good synchrony within 36–48 h (Hütterman, 1973); however, there have been no reports on the synchronous transformation of plasmodia into cysts except for the previous description reported by us (Hattori and Kaneko, 1986). We have studied the function of an active phosphatase by working with a plasmodium (a macroplasmodium). Studying the function of phosphatases in macroplasmodium during sclerotium formation has several additional merits, compared to microplasmodium, e.g.: (1) precultures on agar plates with oats can select the front of a plasmodium which is a very vigorous portion of the plasmodium and the front portion can be a good starting material for the fairly synchronous induction of sclerotia, (2) cultures on agar plates have advantages in avoiding contamination by microorganisms, (3) harvesting of macroplasmodia is easier than microplasmodia. In this report we show the behavior of a

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phosphatase under dark-starvation, and we suggest that one of the two isoforms detected just before the sclerotization initiates the formation of sclerotium in plasmodia.

2. Results and discussion

2.1. Sclerotization of plasmodia under dark-starvation: morphological observation

Plasmodia successively cultured on 1.5% agar plates containing semi-defined medium were transferred onto one end of the 1.5% water agar plate (pH 8.0), whereas at the other end sterile oats were evenly spread. Plasmodia, which had been bright yellow colored and had migrated rapidly to the oat flakes, were selected for use in dark-starvation experiments.

Within about 2 days after dark-starvation treatment, more than 70% of the plasmodia formed sclerotia (Fig. 1); however, no sclerotia were observed within $1\frac{1}{2}$ days. At 1.5 days after dark-starvation treatment, approximately 95% of the plasmodia plates had formed sclerotia. These sclerotia were quite dry and no protoplasmic streaming was observed in the cytoplasm of any of the sclerotia. Moreover, plasmodia had formed complete sclerotia when observed by scanning electron microscopy as shown in Fig. 2a. Small convex shaped protrusions (spherules) were also distributed on the surface of a sclerotium, compared with the relatively smooth surface of the plasmodia (Fig. 2b). Each sclerotium protrusion (spherule) was composed of a cytoplasm and a cell wall which enclosed the cytoplasm (Fig. 2c).

It was previously observed that sclerotia prepared from microplasmodia under starvation conditions were comprised of numerous spherules, where each individual spherule was itself a separate unit with its own cell wall (Chet and Kislev, 1973). The small globular shaped protrusions distributed on the surface of the sclerotium seem to be identical structures to those of the spherules described previously (Chet and Kislev, 1973).

At a higher magnification the structure of a spherule prepared from macroplasmodium, which is composed of a unit of cytoplasm and a cell wall, is clearly shown for the first time. Observation of the surface of sclerotia by scanning electron microscopy provided evidence that the sclerotia formed from plasmodia under dark-starvation treatment in this study maintained the complete characteristic structures of sclerotia prepared from microplasmodia by starvation as described previously (Chet and Kislev, 1973).

As shown in Fig. 1 the dark-starvation treatment reduced the fresh weight of plasmodium per plate directly by 50% within 1.5 days of the starvation period. Fresh weights dropped to levels of *ca.* 15% after 3 days of dark-starvation.

High frequency of sclerotium formation appears to require a certain initial fresh weight of the plasmodia. When *ca.* 13 mg fresh weight of plasmodium on the original section per plate was transferred to 1.5% agar plates containing 60 mM NaCl, a high frequency of *ca.* 95% sclerotization was attained, indicating that the optimum fresh weight of plasmodium was around *ca.* 13

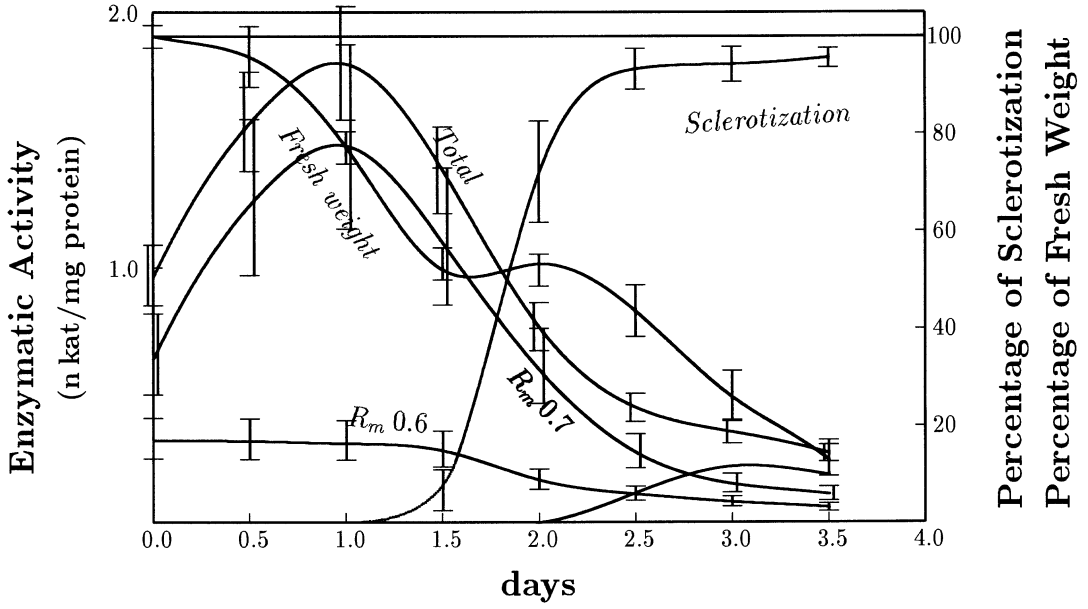

Fig. 1. Rate of sclerotization of plasmodia in *Physarum polycephalum* and behavior of phosphatase isoform activities after the period of dark-starvation under the starvation condition yielding *ca.* 95% sclerotization. The number of plates used was 302. The percentages of each enzymatic activity of the four isoforms were calculated as the ratio of each peak height of enzymatic activity, and the values of activity were determined as proportions of total activity applied to each lane.

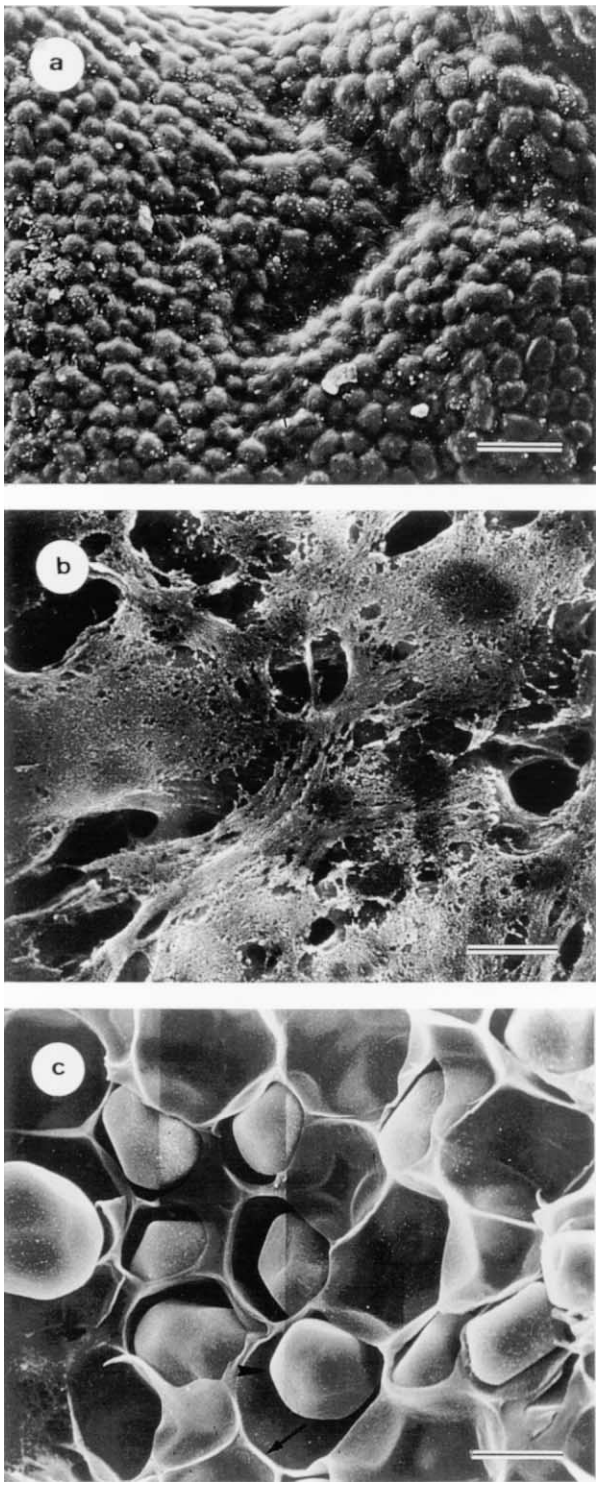

Fig. 2. Morphology of the sclerotium. (a) Scanning electron micrograph of a sclerotium. A representative sclerotium formed from a plasmodium at about 2.5 days after dark-starvation treatment. Small globular shaped protrusions (spherules) are distributed on the surface of a sclerotium (×300), bar, 50 μm. (b) Scanning electron micrograph of a plasmodium (×3000), bar, 5 μm. (c) Scanning electron micrograph of a fracture of a sclerotium. A representative sclerotium formed from a plasmodium at about 2.5 days after dark-starvation treatment. Each protrusion (spherule) of a sclerotium was composed of a cytoplasm and a cell wall, which enclosed the cytoplasm (×1500), bar, 10 μm.

mg in our preliminary experiments. For synchronous formation of sclerotium from plasmodium under dark-starvation conditions, the 50% reduction in the fresh weight of plasmodium per plate within 1.5 days of starvation was essential to achieve *ca.* 95% sclerotia formation. When the fresh weight of plasmodium per cell was reduced to the level of *ca.* 75% within one and a half days of starvation, the rate of sclerotia formation was only *ca.* 20% (data not shown).

2.2. Changes in phosphatase activity during sclerotia formation

Hydrolytic activity toward *p*-nitrophenyl phosphate (*p*-NPP) at pH 7.6 was not detected either in plasmodia or sclerotia (data not shown), as reported previously (Kaneko and Yamaura, 1982). In a microplasmodium, strain *M3C*, no phosphatase is detected under optimal pH in the alkaline region (Hütterman et al., 1970).

As shown in Fig. 1, the specific activity of the enzyme increased markedly by *ca.* two-fold within the first day of treatment. After the first day of starvation, enzymatic activity decreased rapidly to a level lower than the initial level within two days of the starvation period.

Previously, the specific activities of an acid phosphatase were assayed during growth in semi-defined medium and during differentiation induced by starvation using *Physarum polycephalum* strain M_3CIV (Hüttermann et al., 1979). The greatest increase in specific enzymatic activity in the starvation system was observed at the time when the first spherules were being formed with an increase of *ca.* 95%.

The results obtained in the present study agree approximately with the increase of enzymatic activity observed in the starvation system described previously (Hüttermann et al., 1979); however, the maximum enzymatic activity observed in the starvation system was observed at 12 h before the first sclerotia were being formed. The method of plasmodial sclerotization induction was established to obtain a high degree of synchrony as shown in Fig. 1. The nearly two-fold increase in enzymatic activity after 1 day of starvation, followed by a decrease below original levels after 3.5 days of dark-starvation, showed for the first time the precise profiles of phosphatase activity during plasmodial sclerotization. This suggests that the reduction in fresh weight of plasmodia is essential for increasing in enzymatic activity.

2.3. Analysis of phosphatase isoforms behavior during sclerotia formation by polyacrylamide gel electrophoresis

As shown in Fig. 3, electrophoresis in native conditions of the enzyme preparation of plasmodial phosphatase grown in 1.5% agar plates containing semi-defined medium showed two distinct enzyme activity

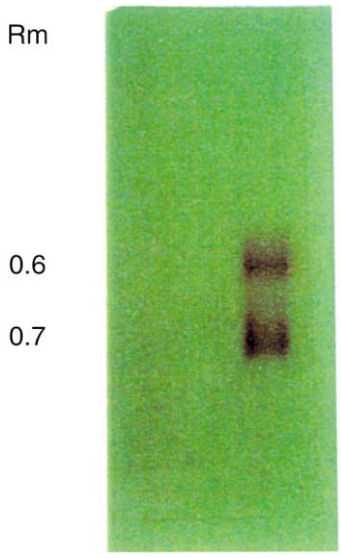

Fig. 3. Polyacrylamide gel electrophoresis of *Physarum* plasmodial phosphatase. Aliquots of 20 μl (46 μg protein/lane) was applied to a polyacrylamide gel. Bands representing phosphatase isoforms were localized using Fast Blue B salt and α-naphthyl phosphate as substrate.

bands, suggesting that the phosphatase preparation consists of at least two isoforms (*Rm* values of 0.6 and 0.7). Densitometric scanning profiles of each isoform was surveyed during dark-starvation under the starvation yielding *ca*. 95% sclerotization conditions (Fig. 4). After 2.5 days of dark-starvation, two additional isoforms, the third and fourth isoforms (*Rm* values of 0.5 and 0.4) were detected.

In order to determine which among the isoforms of *Rm* values of 0.6 or 0.7 caused the increase of enzymatic activity during the first days of dark-starvation shown in Fig. 1, the behavior of each isoform activity under plasmodial sclerotization was calculated from densitometric scanning data (Fig. 4) of polyacrylamide gel electrophoretograms from enzyme preparations during dark-starvation. The horizontal axis of the lane profiles represents the distance of migration by the bands exhibiting enzymatic activity, and the vertical axis represents enzymatic activity. After the percentage of enzymatic activity of each two isoforms was calculated, as the ratio of each peak height of enzymatic activity, the values for

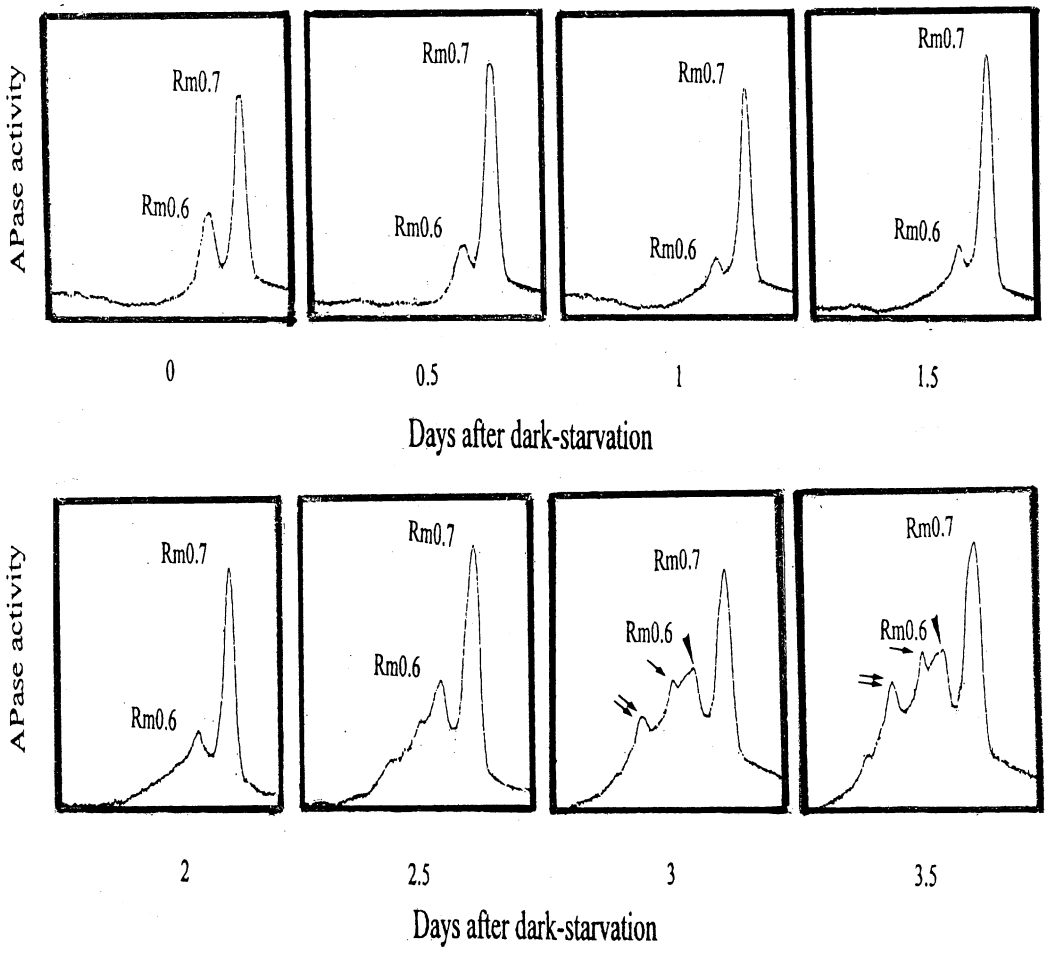

Fig. 4. Densitometric scanning of polyacrylamide gel electrophoretograms of plasmodia phosphatase during dark-starvation under the starvation condition yielding *ca*. 95% sclerotization. In lane profiles, the horizontal axis of the lane represents the distance of migration by bands of exhibiting enzymatic activity, and the vertical axis represents enzymatic activity. The third and fourth isoforms (*Rm* values of 0.5 and 0.4) are indicated by arrows and double arrows, respectively.

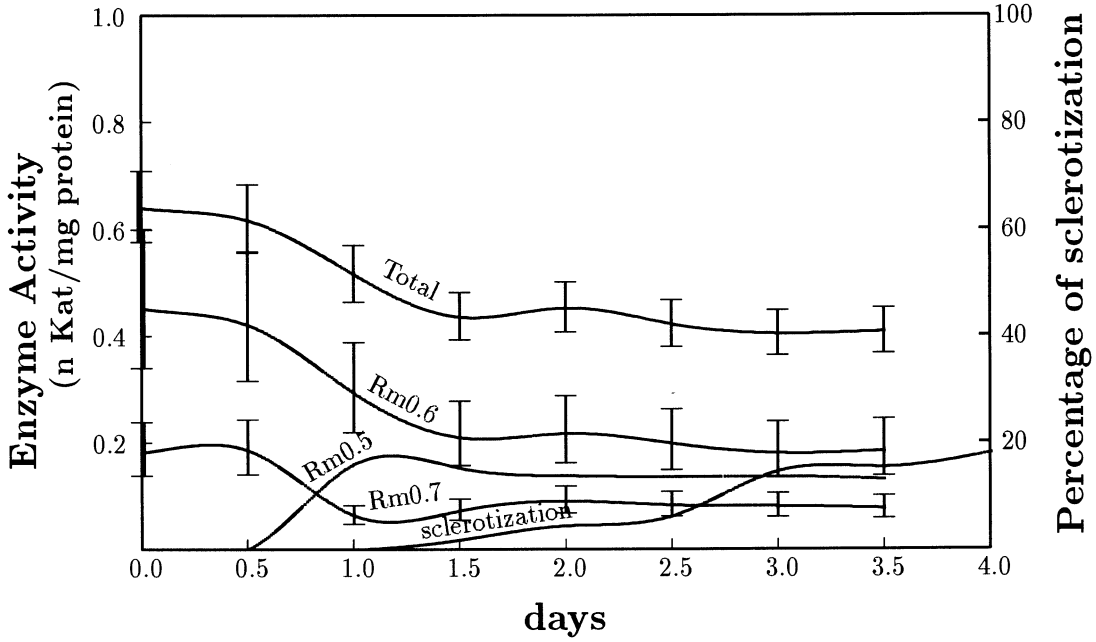

Fig. 5. Rate of sclerotization of plasmodia in *Physarum polycephalum* and behavior of phosphatase isoform activities after the period of dark-starvation under the starvation condition yielding *ca.* 20% sclerotization. The number of plates used was 178. Every 12 h after beginning dark-starvation conditions of plasmodial cultures, 20–25 plates of plasmodia were taken out at random from the incubator and the number of plates on which plasmodia had formed sclerotia were counted.

each activity were found to be proportional to the total activity applied to each lane. As shown in Fig. 1, after beginning dark-starvation, the enzymatic activity of isoform (*Rm* value of 0.6) decreased gradually within 1.5 days of starvation. It was followed by a monotonous decrease in activity to less than 20% of the initial activity by the end of the observation period. The enzymatic activity of isoform (*Rm* value of 0.7) activity increased up to *ca.* two-fold within the first day of the starvation period. Thereafter it decreased monotonously to levels less than that of the first 2 days of starvation.

When *ca.* 9.6 mg fresh weight of plasmodium on the original section was transferred to 1.5% agar plates containing 60 mM NaCl, a relatively high frequency of *ca.* 80% sclerotization was obtained (data not shown), while under the starvation conditions where *ca.* 7.0 mg fresh weight of plasmodium on the original section per plate was transferred to agar plates, a low frequency of *ca.* 20% was obtained (Fig. 5). Under the starvation conditions yielding *ca.* 80% sclerotization, the behavior of each enzymatic activity for both isoforms coincided very closely with the enzymatic activities under starvation conditions which produced *ca.* 95% sclerotization (data not shown). Starvation conditions yielding only 20% sclerotization showed that the initial level of the specific activity of the acid phosphatase was *ca.* 64% lower than that as compared with the condition yielding *ca.* 95% sclerotization (Figs. 1 and 5) and that the enzymatic activity decreased to a level *ca.* 72% lower than the initial level within 1.5 days of the starvation period. Under the starvation conditions yielding *ca.* 20% sclerotization the enzymatic activity of isoform (*Rm* value of 0.7) was lower than the isoform (*Rm* value of 0.6), and decreased linearly to less than 45% of the initial value (Fig. 5). After 1 day of dark-starvation, one additional isoform, the third isoform (*Rm* value of 0.5), was detected, though the fourth isoform (*Rm* value of 0.4) was not.

The behavior of isoform (*Rm* value of 0.7) strongly suggests that it plays a leading role in plasmodium formation of sclerotium under dark-starvation. The role of the isoform warrants further examination related to the cessation of cytoplasmic streaming in plasmodium, since cytoplasmic streaming stops in plasmodia upon the formation of sclerotia.

3. Experimental

3.1. Plant material and culture method

Plasmodia of *Physarum polycephalum* (strain Ng-1 supplied by Professor K. Kohama, Gunma University) were used. For successive cultures, plasmodia were placed on 1.5% water agar plates containing a semi-defined medium described by Kuroda et al. (1988) and modified by Kohama et al. (personal communication). The medium contained 2% soytone peptone (BECTON DEIKINSON, MD, USA), 2% sucrose, 5 μg/ml hemine, 16.8 mM citric acid monohydrate, 0.6 mM EDTA (disodium salt), 15 mM KH_2PO_4, 9.2 mM $CaCl_2$,

2.4 mM $MgSO_4$, 0.2 mM $FeCl_2$, 0.12 mM $ZnSO_4$, 0.2 mM biotin and 2 mM thiamine, pH 4.6, at 25 °C in the dark.

3.2. Preparation of plasmodia for dark-starvation experiments

Induction of plasmodial sclerotization in high synchrony as described previously (Hattori and Kaneko, 1986) was performed in this experiment with slight modifications. After plasmodia of approximately 0.1 g fresh weight were inoculated to the center of 1.5% water agar plates, pH 5.8 in a Petri dish ($\phi = 9$ cm), about 3 g of sterile oat flakes were spread evenly over the plate. After 1 day incubation, equal portions of a new plasmodium along with its underlying substratum were cut into 8 sections of equal size. Each section was placed in the center of another 1.5% agar plate containing 60 mM NaCl and incubated for 16–18 h. After the plasmodium expanded to cover the entire agar surface, the original inoculum sections were then removed. Within 3–4 h the remaining plasmodia migrated back into the central blank space and fused, resulting in the formation of plasmodial disks of 8.5 cm in diameter. The plasmodia were again cut, together with their agar supports, into quarters and placed onto new plates. After the plasmodia migrated from the original inoculum sections to new plates, the original inoculum sections were removed. Plasmodia which migrated from the original quarter sections were incubated and dark-starved. All procedures were conducted at 25 °C. Every 12 h after beginning dark-starvation conditions of plasmodia cultures, 33–34 plates of plasmodia were taken out at random from the incubator and the number of plates on which plasmodia had formed sclerotia were counted. Sclerotium formation was examined by two methods: (1) observation of the ceasing of cytoplasmic streaming under a microscope; (2) touching the surface of the plasmodium by a sterile spatula to examine whether it has become a dry crust.

3.3. Enzyme extraction

Enzyme extraction was carried out by the method described previously (Kaneko and Kato, 1990). All steps were carried out at 0–4 °C. Plasmodia (*ca.* 100 μg fresh weight) and sclerotia (*ca.* 50 μg fresh weight) were homogenized in Teflon-glass homogenizer at 1400 rpm in *ca.* 10 ml of 20 mM Tris-HCl, pH 7.2, 0.3% 2-mercaptethanol, 35% (w/v) sucrose, and 3 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid for 5min at 0–4 °C. Homogenates of plasmodia and sclerotia were centrifuged at 1400 g for 60 min. The supernatant was brought to 75% saturation with $(NH_4)_2SO_4$. After being left for 1 h, the precipitate was collected by centrifugation at 20,000 *g* for 1 h and dissolved in 2 ml of 50 mM Tris–HCl, pH 7.2. The solution was dialyzed against the same buffer. The dialysate was called "the enzyme preparation".

3.4. Polyacrylamide gel electrophoresis

Electrophoresis in a non-denaturating buffer was performed on 7.5% (w/v) polyacrylamide slab gels at 4 °C according to the method of Davis (1964). Bands representing phosphatase activity were localized using Fast Blue B salt and α-naphtyl phosphate disodium salt as a substrate. Developed gels were stored in 7.5% acetic acid. Electrophoretograms of enzyme preparations were photographed and the images were saved on floppy disks, using a monochrome CCD camera and image saver (AE-6910 Printograph, ATTO Corp., Tokyo). The densitometric scanning was indirectly obtained using the lane profiles from floppy disk images. Saved images were analyzed with imaging resolution software (Lane Spot Analyzer AE-6920 WLSA, ATTO Corp., Tokyo) on a personal computer (PC-9821V12, NEC Computer Co., Tokyo).

3.5. Enzymatic activity and protein concentration assay

Every 12 h after initiating dark-starvation of plasmodia, 33–34 plates of plasmodia were taken at random from the incubator as samples during the dark-starvation period over 3.5 days, when almost all the plasmodia undergoing dark-starvation had formed sclerotia. After enzyme extraction was carried out on each sample, the hydrolytic activity of the enzyme preparation toward *p*-nitrophenyl phosphate (*p*-NPP) at pH 5.0 was assayed.

Phosphatase activity was assayed using 3.3 mM *p*-NPP and 67 mM sodium acetate buffer, pH 5.6, in a total volume of 1.5 ml at 35 °C. The reactions were terminated by adding 1.5 ml of 2 M Na_2CO_3 solution and the amount of *p*-nitrophenol released was determined spectrophotometrically at 405 nm. Protein concentration was determined by measuring A_{280} (optical density at 280 nm) with bovine serum albumin as a standard.

3.6. Scanning electron microscopy

Plasmodia and sclerotia were rinsed in 0.1 M potassium phosphate buffer, pH 5.8, containing 0.4 M mannitol (KPBM) and were fixed at 4 °C with 1.25% glutaraldehyde in KPBM for 3 h, followed by rinsing in distilled water, and post-fixed in 1% OsO_4 in distilled water at 4 °C for 2 h, according to a previously described method (Osumi and Torigata, 1977) with modifications. The specimens were then rinsed with 50% acetone and dehydrated in increasing concentrations of acetone, substituted with isoamyl acetate, and dried by critical point drying (Hitachi Critical Point Dryer HCP-1, Tokyo). After being dried, the specimens were sputter-

coated with gold and palladium and were examined with a scanning electron microscope (SEM) S-430 at 15 kV.

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