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Biochemistry of Fern Spore Germination: Protease Activity in Ostrich Fern Spores[1]

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ABSTRACT

Protease activities were detected in quiescent and germinating spores of the ostrich fern (*Matteuccia struthiopteris* [L.] Todaro). Peak endopeptidase, aminopeptidase, and carboxypeptidase activities were detected 12 to 24 hours after spores began imbibing under light. There was a correlation between activities of proteases, the onset of a decline in levels of soluble protein, and an increase in levels of free amino acids. The earliest visible event of spore germination, breakage of the spore coat and protrusion of a rhizoid cell, was observed after peak protease activity, 48 to 72 hours after the start of imbibition. Results of this study demonstrate similarities in the pattern of protease activities during germination of ostrich fern spores to those of some seeds.

Similarities exist between the storage reserve metabolism of seeds and fern spores. Both may contain reserves of protein, lipid, phytic acid, and sugars (1, 2, 8, 9, 23, 24). Storage proteins are a storage source of amino acids for *de novo* protein synthesis, and are presumed to sustain germination and early post-germinative growth (1). Ordinarily, storage proteins are found in protein bodies and are hydrolyzed by concerted activity of several classes of proteolytic enzymes, such as EPs[3], APs, and CPs. Protease activity in germinating seeds has been studied extensively (14, 16, 21), but the only class of protease identified in ferns are the APs (6, 12, 22).

Details of protease activity during the processes of fern spore germination and early fern prothallial development are needed to understand how storage protein reserves of fern spores are catabolized. Since cellular concentrations of amino acids may be regulated by the activity of proteases (27), knowledge about proteases may also lead to a better understanding of protein synthesis and protein turnover during fern spore germination and prothallial development. This study describes, the concerted activity of EPs, APs and CPs during fern spore germination, and correlates these activities with levels of soluble proteins, free amino acids, and with morphological stages of development.

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[3] Abbreviations: AP, aminopeptidase; Bz, α-*N*-benzoyl, CBZ, carbobenzoxy; CP, carboxypeptidase; EP, endopeptidase; NEDD, (*N*-1-naphthyl)ethylene diamine dihydrochloride; NHNan, *p*-nitroanilide; NHNap, 2-naphthylamide; RuBPCase, ribulose-1,5-bisphosphate carboxylase/oxygenase.

MATERIALS AND METHODS

Chemicals. α-Aminoacyl NHNan substrates were purchased from Sigma Chemical Co. or from Vega Biochemicals. Ninhydrin was purchased from U.S. Biochemical Corp., and diethylbarbituric acid from Amend Drug and Chemical Co. All stock chemicals were certified reagent grade from Fisher Scientific Co. All amino acids and amino acid substrates were L-isomers except for Bz-Arg-NHNap, which was a mixture of D- and L-isomers.

Plant Material. Sporophylls of the ostrich fern (*Matteuccia struthiopteris* [L.] Todaro) were collected in mid-November in Hanover, NH. They were soaked in tap water for 15 to 30 min and then air dried in the laboratory, causing spores to be released. Contaminating sporangial debris was removed by sifting through a series of wire mesh filters decreasing in mesh to 75 μm pores. Spores were stored in a light proof container at 4°C and used within 2 years of collection.

Culture Conditions. To initiate the germination processes, 0.2 to 3 g of spores were suspended in 100 to 500 ml of distilled, deionized H_2O, placed in an environmentally controlled room at 23 ± 2°C, and exposed to 12 h of light at 40 μE m^{-2} s^{-1} PAR provided by a combination of incandescent and fluorescent bulbs.

Homogenization of Spores. Spores were collected prior to homogenization by suction filtration on a Büchner funnel. A variety of mechanical abrasive techniques were utilized to homogenize spores. These techniques were all equally effective and using more than one at a time permitted us to process multiple spore samples. They include a Tenbroek ground glass mortar and pestle for 1 h (total protein, soluble protein and ninhydrin positive compounds), a Waring Blendor with sea sand as an abrasive for 10 min (EP, spore protein substrate), a Polytron tissue homogenizer at a setting of eight for 15 min (AP), and a Bead Beater tissue homogenizer for 15 min (CP). Ordinarily a 1:10 ratio of spores to buffer was used. Homogenates were centrifuged at 20,000*g* for 20 min (total protein soluble protein, ninhydrin positive compounds, AP, CP), or for 40,000*g* for 10 min (EP, spore protein substrate), and the supernatant fluids were assayed as indicated. Assay conditions were optimized to produce maximum reaction rates. All extractions were done at 4°C.

Free Amino Group Determinations. Ninhydrin positive materials in 200 mg of spores were measured at various times during the processes of germination by the method of Yemm and Cocking (28), using leucine as the standard.

Protein Determination. Total protein of quiescent spores was determined by homogenizing 100 mg of spores in 20% (w/v) TCA. After centrifugation the pellet was extracted with 0.3 N NaOH at 37°C for 16 h, and the mixture centrifuged at 20,000*g* for 20 min. The supernatant fluids from both centrifugation steps were assayed for protein by the method of Lowry *et al.* (15).

Soluble protein was determined from extracts of 100 mg of spores that were cultured and collected as described above.

Homogenization was done in 0.2 M sodium phosphate (pH 7.5). The supernatant fluid was assayed for protein (15).

Endopeptidase Activity. EP activity was extracted from 1 g spores in 0.2 M sodium phosphate buffer (pH 6.75) with 300 mg insoluble PVP. Assays utilized an artificial peptide substrate (Bz-Arg-NHNap) and an endogenous spore protein. The protein was extracted from quiescent spores in 0.2 M sodium phosphate buffer (pH 7.5). The filtered and centrifuged extract was layered on a 10% PAGE performed according to Laemmli (13), omitting SDS. A prominent anodal migrating protein was selected for use. The protein was sliced from the gel, minced in buffer, and dialyzed against several changes of 0.2 M sodium phosphate (pH 6.75) for 2 d and used in the assay. When the recovered protein was subjected to SDS-gel electrophoresis (13), a single polypeptide band was detected with a M_r of 14,500. A 0.05 ml of extract was mixed with 0.95 ml solution containing 0.5 mg endogenous spore protein and incubated at 37°C for 1 h. The reaction was terminated with TCA to a final concentration of 10% and left at room temperature for 15 min. The mixture was centrifuged in a Beckman microfuge for 5 min, the supernatant was decanted, neutralized with NaOH, and then assayed for protein according to Bradford (4). One unit of activity represents the quantity of enzyme necessary to produce an A_{595} equivalent to 10 μg BSA-dye complex in 1 min. The solution also was assayed for ninhydrin positive materials (26).

For assays using the synthetic substrate, 0.1 ml aliquot of extract was mixed with 0.9 ml of Bz-Arg-NHNap (0.4 mg/ml dissolved in 0.2 M sodium phosphate buffer, pH 6.75) and incubated at 37°C for 15 min or 30 min. The reaction was terminated with 0.5 ml of 40% (w/v) TCA, and left at room temperature for 10 min. The 2-naphthylamine liberated from Bz-Arg-NHNap was diazotized to NEDD, and the colored dye was quantitated at 520 nm, using 2-naphthylamine as the standard (10). One unit of enzyme activity is the quantity of enzyme necessary to hydrolyze 1 μmol of substrate in 1 min at 37°C under conditions of the assay.

EP activity was labile, unlike AP and CP activity. It was not detected when PVP was omitted from the extraction medium. Once extracted, EP activity declined rapidly, and was completely lost in 6 d, whether stored at 4, −20 and −80°C, or as an 85% $(NH_4)_2SO_4$ pellet.

Aminopeptidase Activity. Spores (1 g) that were cultured and collected as above were homogenized in 12.8 mM borate-barbital buffer, pH 7 (11). α-Aminoacyl NHNan substrates were solubilized in 1 ml of DMSO and diluted to 10 ml with 12.8 mM borate-barbital buffer (pH 7). The assay was performed by incubating 0.05 ml of extract with 1 ml of substrate at 37°C. After 3.75 min the reaction was terminated by the addition of 0.25 ml of 20% (v/v) acetic acid. The solution was centrifuged at 1,000*g* for 15 min and the A_{410} was measured on a Gilford 2000 spectrophotometer. Activity was calculated using an experimentally derived molar absorbtivity for *p*-nitroaniline of ϵ_{5174}, which is appreciably lower than the corresponding value in aqueous solution (26). One unit of activity is the quantity of enzyme required to hydrolyze 1 μmol of substrate in 1 min at 37°C under conditions of the assay.

Carboxypeptidase Activity. Three g of spores, cultured and collected as noted above, were homogenized in 25 mM citrate-50 mM Na_2HPO_4 buffer (pH 5). The spore extract was dialyzed overnight against CP homogenization buffer. A precipitate which formed overnight was removed by centrifugation. In a typical assay, 18.5 mg of CBZ-Phe-Ala was dissolved in 5 ml of DMSO, and diluted to 50 ml with 25 mM citrate-50 mM Na_2HPO_4 buffer (pH 7.4), containing 0.5 M NaCl. The reaction was initiated by the addition of 0.1 ml of the extract to 0.9 ml of CBZ-Phe-Ala solution which was equilibrated at 37°C. The reaction was terminated after 3 h with 0.1 ml of 20% (w/v) TCA, and 0.05 ml aliquots were assayed for free alanine (28). Units of activity are defined as the amount of enzyme required to release 1 μmol of alanine in 1 min at 37°C under the conditions of the assay.

RESULTS AND DISCUSSION

Morphological Observations. The spores swelled to an elliptical ovoid shape within minutes of being placed in water. Germination, determined by protrusion of a rhizoid, was observed 48 h to 72 h after spore imbibition began (Fig. 1). By 144 h the fern prothalli had developed into two to three celled filaments. The developmental pattern described here is identical with prior descriptions of ostrich fern spore germination (9). Other fern spores germinate in similar patterns, except that the timing often is different (17, 20).

Protein and Free Amino Group Levels. Quiescent ostrich fern spores contained 21% protein by wet weight, of which 83% was TCA insoluble. Fifty-six percent of total protein (12% of the wet weight of the spores) was soluble in 0.2 M sodium phosphate buffer (pH 7.5). Ninhydrin positive material (presumably free amino acids) comprised 3% of the wet weight of quiescent spores.

There was a decline in levels of soluble protein and an increase in the levels of free amino acids as the spores imbibed, germinated, and the prothallial filaments began to elongate (Fig. 1). During the first 12 h after being sown, changes in the levels of soluble protein or free amino acids were not detectable. The most rapid decline in soluble protein levels was detected between 12 and 36 h. Free amino acid increased most rapidly between 12 and 72 h. As the fern prothallial filaments began to elongate, levels of soluble protein and free amino acids began to plateau.

Spores were not supplied with exogenous N, thus storage proteins were the only major source of N during germination. The increase in free amino acids is best explained by the concerted action of EPs, APs, and CPs, which released short peptides and free amino acids from storage proteins into solution.

Endopeptidase Activity. EP activity, first detected in quiescent and germinating spores when Bz-Arg-NHNap was the substrate, was maximal when extraction and assays were done at pH 6.75. Bz-Arg-NHNan, Bz-Tyr-NHNap, Bz-Phe-NHNap were not hydrolyzed, despite their structural similarities to Bz-Arg-NHNap (see also Table I).

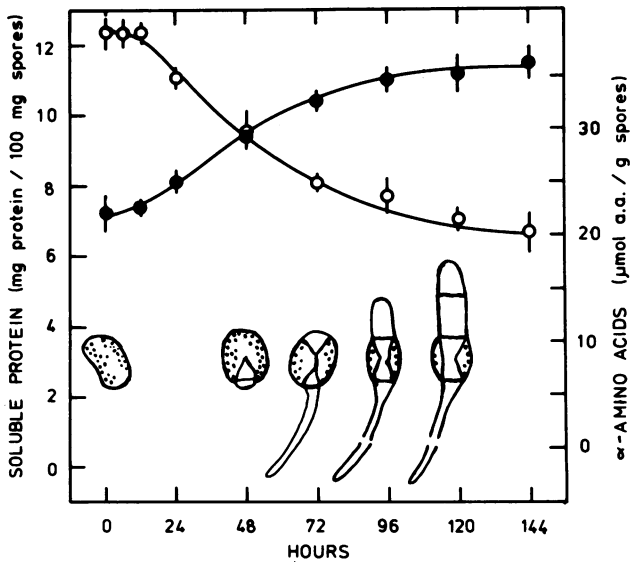

FIG. 1. Soluble protein and free amino acid levels in spores during imbibition, germination, and prothallial filament development. The morphological stages of development that are diagrammed on the bottom represent brightfield microscopic (×125) observations (O) soluble, (●) free amino acids. Bars represent the SE.

A broad peak of EP activity against Bz-Arg-NHNap was observed between 6 and 48 h (Fig. 2). By 72 h this activity had declined to 30% to 40% of the maximum value.

Since germinating spores could contain EPs which cannot hydrolyze Bz-Arg-NHNap, an endogenous spore protein was also used as substrate for *in vitro* assays. The endogenous protein used for assay has a polypeptide M_r of 14,500, but was not characterized further. However, in spores of another fern, *Adiantum capillus-veneris*, a M_r 14,400 polypeptide was identified as a prominent storage protein (18). In initial experiments, the ostrich fern spore protein digest was assayed for an increase in soluble peptides by the dye-binding technique of Bradford (4), and for an increase in levels of ninhydrin positive material (28), which measure principally large peptides, and the free amino groups of amino acids or small peptides, respectively. Levels of ninhydrin positive compounds did not increase appreciably even when enzyme-substrate incubation was extended to 6 h, indicating that few free amino groups were generated during digestion. However, increased levels of soluble dye-binding compounds were detected after 0.5 h of incubation. Consequently, the increase in dye-binding compounds was due principally to large soluble peptides, and not to free amino acids or small peptides.

EP activity toward the endogenous protein was present in quiescent spores, and maximum EP activity was detected between 6 and 24 h (Fig. 3). Activity levels declined to 20 to 30% of maximum by 48 h, and then remained constant up to 144 h. In contrast to the results obtained with this native protein substrate, EP activity at 48 h toward Bz-Arg-NHNap remained high (Fig. 2), indicative of multiple proteases of different specificities.

In vitro proteolysis of the endogenous protein demonstrates the presence of at least one EP capable of hydrolyzing native spore protein. Endogenous proteins have been used by others to help determine the function of an EP. Baumgartner and Chrispeels (3) utilized vicilin, the major storage protein of mung beans, to determine the role of the predominant mung bean EP in the degradation of mung bean storage proteins during germination. In a similar manner, Dalling *et al.* (7), utilizing purified RuBPCase, showed that chloroplasts contain EPs capable of regulating RuBPCase *in situ*.

Aminopeptidase Activity. AP activity in quiescent spores was assayed using different α-aminoacyl NHNans. Highest specific activity was obtained with Phe-NHNan. There was a positive correlation between the hydrolysis of a substrate (Table I) and

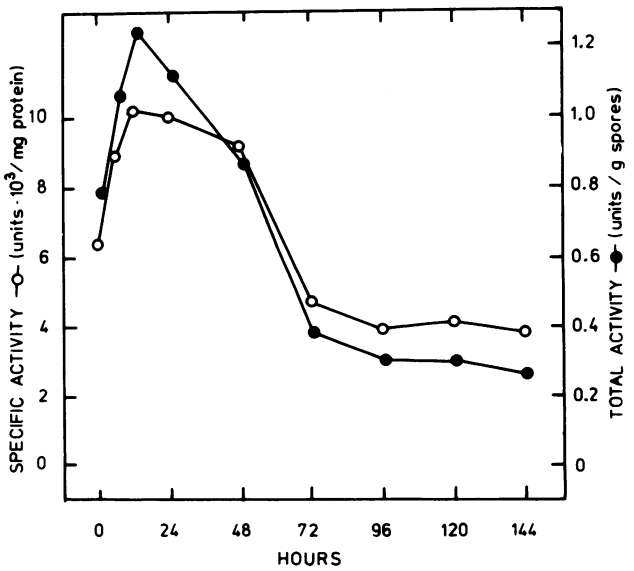

FIG. 2. Time course of EP activity, utilizing BZ-Arg-NHNap as substrate.

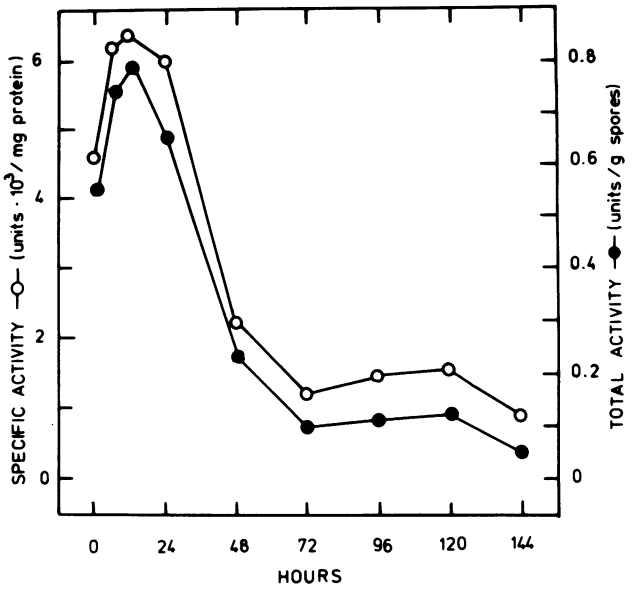

FIG. 3. Time course of EP activity, utilizing the extracted endogenous spore protein as substrate.

Table I. *Hydrolysis of Synthetic Substrates by Extracts from Quiescent Spores*

The specific AP activity of quiescent spores was determined with different NHNan substrates. Excluded from this table are substrates that were not hydrolyzed: acetyl-Ala-NHNan, acetyl-Phe-NHNan, benzoyl-DL-Arg-NHNan, benzoyl-Tyr-NHNan, benzyl-Cys-NHNan, CBZ-Phe-NHNan, Gln-NHNan. Spores were extracted and assayed as described in "Materials and Methods."

Substrate	Specific Activity ± SE	Maximum Activity
	units·mg protein^{-1}	%
Phe-NHNan	171 ± 7	100
Leu-NHNan	65 ± 5	38
Pro-NHNan	38 ± 5	22
Ala-NHNan	33 ± 8	19
Gly-NHNan	12 ± 3	7
Cys-NHNan	5 ± 1	3
Arg-NHNan	3	2
His-NHNan	3	2

the nonpolarity of its amino acid moiety (19). None of the NHNan substrates with blocked amino termini were hydrolyzed, indicating that the hydrolysis of α-aminoacyl NHNan substrates depends on the presence of a free amino group (Table I).

The optimal pH for AP activity, determined with Phe-NHNan and with Leu-NHNan was pH 7. Levels of AP activity were barely detectable below pH 5 or above pH 10. Only one peak of activity was detected in the spores at any of the time intervals assayed (pH 7), from quiescence until 144 h after the spores were sown in water. The time course for AP activity during spore imbibition, germination, and early prothallial development is shown in Figure 4. AP levels in spores increased rapidly during the first 12 h of imbibition, and then declined rapidly between 12 and 72 h. When germination occurred, the level of total AP activity had declined to 50% of the maximum activity observed at 12 h. After 144 h, total activity had declined to 35% of maximum activity and specific activity was equivalent to the specific activity in quiescent spores.

Carboxypeptidase Activity. Preliminary experiments indicated that highest specific CP activity was detected when the pH of the extraction medium was 5.0 and the pH of the assay was 4.6.

The time course of CP activity was determined from spore

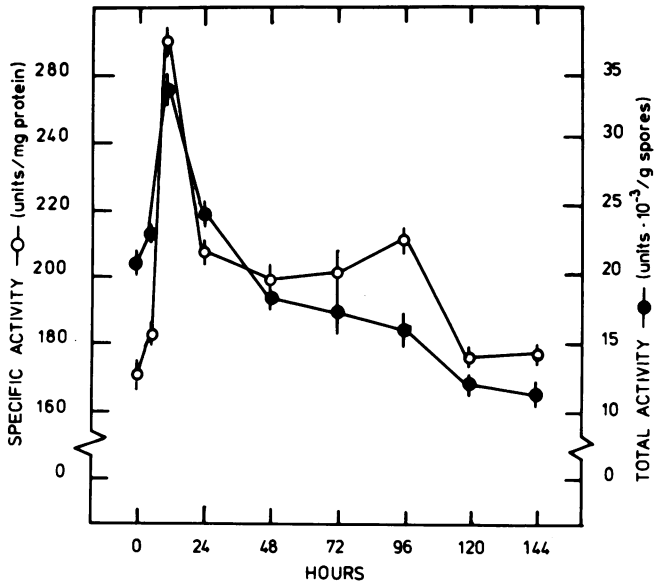

FIG. 4. Time course of AP activity. Bars represent the SE.

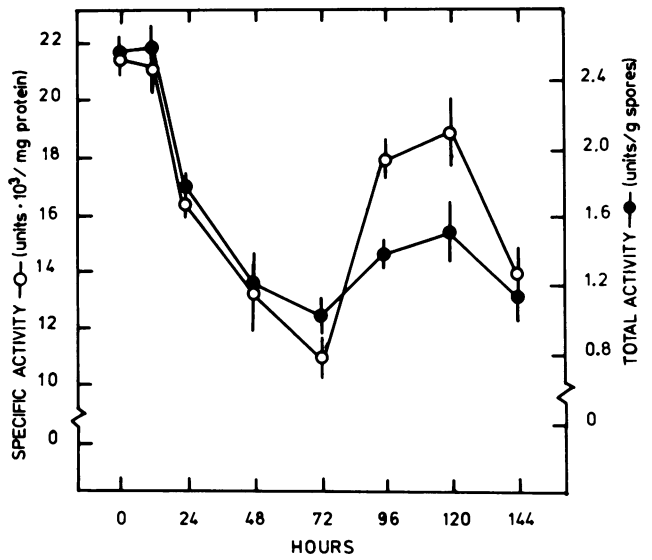

FIG. 5. Time course of CP activity. Bars represent the SE.

imbibition through the stages of germination and early fern prothallial development (Fig. 5). Levels of CP activity did not change for the first 12 h after the start of imbibition. After 12 h there was a sharp decline in total activity, with only 36% of the total activity remaining by 48 to 72 h, when most spores germinated. This decline in CP activity ended by 72 h, corresponding to the interval when soluble protein levels declined and free amino acids increased. A second peak in total CP activity was noted 96 to 120 h after the spores had been placed in water, representing a 49% increase from 72 h, but still much lower than the maximum total activity observed at 0 to 12 h. Specific activity at 96 to 120 h was 85% of the specific activity of quiescent spores (Fig. 5). By 144 h total activity had declined to approximately the level observed at 72 h.

Comparison of Activity Profiles. All classes of proteases assayed were present in quiescent spores, indicating the presence of potentially active proteases at the onset of the germination processes. Clearly, these proteases were synthesized during sporogenesis.

Comparing the different protease activity profiles, peak activity of each occurred at approximately 12 h. Changes in levels of soluble protein and free amino acids were detected immediately after peak protease activities, during an interval in which protein body hydrolysis was detected ultrastructurally (2, 5, 9). These temporal changes correlate all three protease activities observed to the breakdown and mobilization of storage protein reserves during ostrich fern spore germination. Similar correlations occur during degradation of storage proteins of many seeds (12, 16, 25), suggesting that the processes involved in protein metabolism in seeds and fern spores may be similar.

Fern spores provide a unique system for studying the germination process. They are unicellular, structurally simpler than seeds, respond to light, temperature and other environmental factors, yet there are no complex tissue interactions in the germinating spore. Our current findings provide evidence for the presence and activity of proteases in fern spores. Future studies are aimed at characterizing the proteases and determining their regulated functions during germination. In other work from this laboratory, we have identified specific storage proteins that are degraded during the time of protease activity (8, 23). The haploid nature of fern species indicates that they may be suitable for exploring the genetic controls operating during proteolysis.

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