PROPERTIES OF PROTEASE DURING THE GERMINATION OF *ANABAENA* SP 310

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ABSTRACT

Protease was isolated from the spores of *Anabaena* sp 310—a nitrogen-fixing blue-green alga and some of the properties were studied. The initiation of spores occurred on the 16th day of the life cycle concomitant with more protease activity. When casein was used as substrate, the enzyme exhibited maximum activity at pH 7 and 9. This shows the presence of neutral and alkaline range proteases in this alga and the enzyme was stable upto 40°C. In the presence of the above substrate, the enzyme exhibited optimum at a high temperature (60°C). Protease activity was also assayed in the presence of different divalent metal ions (Ca^{++}, Hg^{++}, Mg^{++} and Cu^{++}) and urea. At 5 mM concentration, Ca^{++} and Hg^{++} had an inhibitory effect whereas Mg^{++} and Cu^{++} showed stimulation. At 5 mM concentration, urea inhibited the enzyme activity.

INTRODUCTION

STUDIES on proteolytic enzymes have long been a fascinating subject of research. Protease of microbial and animal origin was studied in detail but little information is availabe on blue-green algal and higher plant proteases. Among plant proteases, detailed studies have been made on the sulphydryl enzymes like papain, bromelin and ficin. Proteolytic activity has also been reported in germinating seedling of barley, wheat, rice, maize and green gram as well as in *Bacillus subtilis*[1]. An acid protease was isolated in a homogeneous form and crystallised from the germinating sorghum[2] and bajra seeds[3]. The relationship between the activity of proteolytic enzymes and sporulation in blue-green alga, *Anabaena doliolum* was also observed[4]. In the present study, we report some of the properties of protease activity in the spores of blue-green alga, *Anabaena* sp 310 during the germination period.

MATERIALS AND METHODS

Anabaena sp 310 was obtained from IARI Culture Collection, New Delhi. Axenic and clonal cultures were obtained from single germinating spores. The alga was grown in modified chu. No. 10 medium[5], free of combined nitrogen source. The experimental cultures were incubated in wooden cabinets fitted with fluorescent light emitting a light intensity of 600 lux and maintained at 28 ± 2°C. Bovine serum albumin (BSA) was a product of Sigma Chemical Company, USA, casein, tyrosine, trichloroacetic acid and other required chemicals were of the highest purity obtained from BDH, India.

Developmental morphology: The spore germination of *Anabaena* sp 310 was observed after 48 hr. Cell division occurs inside the spore wall leading to the formation of 2–4 celled filament. As the growth continues intercalary heterocysts were differentiated until a multicellular coiled filament is produced. Filaments of 16-day old cultures started producing oval-shaped spores centrifugally and the percentage of spores increased with the age of culture.

Preparation of the enzyme: Twenty five day old algal culture containing higher percentage of spores was harvested and the enzyme extract (15% wt/vol) was obtained by grinding the algal suspension with glass powder in ice-cold distilled water at 4°C for about 10 min. The algal homogenate was centrifuged at 5000 rpm for 10 min to remove the cell debris and the supernatant was used as the enzyme source. This crude enzyme extract was taken to assay the proteases.

Determination of protease activity by casein digestion method: The activity was determined by following the method of Keay and Wildi[6]. Casein (2 g) was dissolved in 20 ml of 1 N sodium hydroxide at room temperature; 50 ml distilled water was then added and kept in water bath for about 30 min at 40°C. The pH was adjusted to the desired value by dropwise addition of dilute phosphoric acid. Final volume was adjusted to 100 ml with distilled water.

One ml enzyme extract was added to prewarmed 1 ml casein solution (37°C for 15 min) and the reaction mixture was incubated at 37°C for 10 min. The reaction was terminated by the addition of 2 ml of 0.4 N trichloroacetic acid and was allowed to stay at 37°C for 20 min to get the protein precipitated which was separated by centrifugation.

One ml of the above clear supernatant, and 5 ml of 0.4 M sodium carbonate solution was added followed by 1 ml of Folinciocalten reagent. After thorough mixing, the blue colour of the liberated tyrosine was measured at 660 nm in a spectrophotometer (Bausch and Lomb, USA). A calibration curve was prepared with 0–60 μg/ml tyrosine in 0.2 N hydrochloric acid.

The specific activity of the protease was expressed as μg of tyrosine equivalent liberated in 10 min/mg of protein under the assay conditions.

Protein measurements: The concentration of the protein in the enzyme was determined by the method of Lowry *et al*[7] using crystalline bovine serum albumin.

Estimation of carbohydrates: The carbohydrates were estimated with the anthrone reagent by the method of Herbertt *et al*[8].

Estimation of chlorophyll-a and carotenoids: These pigments were extracted in 80% acetone and the optical density of the acetone soluble pigments was measured in Spectronic-20 spectrophotometer. The chlorophyll-a was estimated quantitatively using the formula of Arnon as modified by Mackinney[9]. The carotenoids were estimated by the method of Jensen[10].

Percentage of sporulation studies: The algal culture was observed under the microscope. The time of appearance of the akinete and then akinete frequency were noted on alternate day. Spore frequency was expressed as percentage of cells differentiated to akinetes per hundred vegetative cells.

RESULTS AND DISCUSSION

(a) *Protease activity during germination*: The enzyme activity was estimated at regular intervals after inoculation of spores. The enzyme exhibited gradual increase in its activity and reached maximum on the 16th day and thereafter the activity declined (figure 1). The pH activity profile of the enzyme is shown in figure 1 which indicates the maximum enzyme activity at pH 9. Sporulation in *Anabaena* sp was started on the 16th day of growth period, accompanied by the increase of the protease enzyme activity and other biochemical metabolites (chlorophyll, carbohydrate and proteins). The protease activity and other biochemical metabolites decreased further with the increase of sporulation percentage (figure 1 and table 1).

The biochemical significance of the correlation between degradation rates and certain phases of cell differentiation has been demonstrated in *Bacillus subtilis*[1] and it has been shown that protease production is one of the primary processes associated with spore differentiation. When an organism passed from the vegetative to sporulation phase, a number of new enzymes are synthesized and pre-existing ones are degraded and therefore the activity of the proteolytic enzyme increased just before the commencement of sporulation and decreased further with increase in sporulation. Our results are in agreement with those of Dikshit *et al*[4].

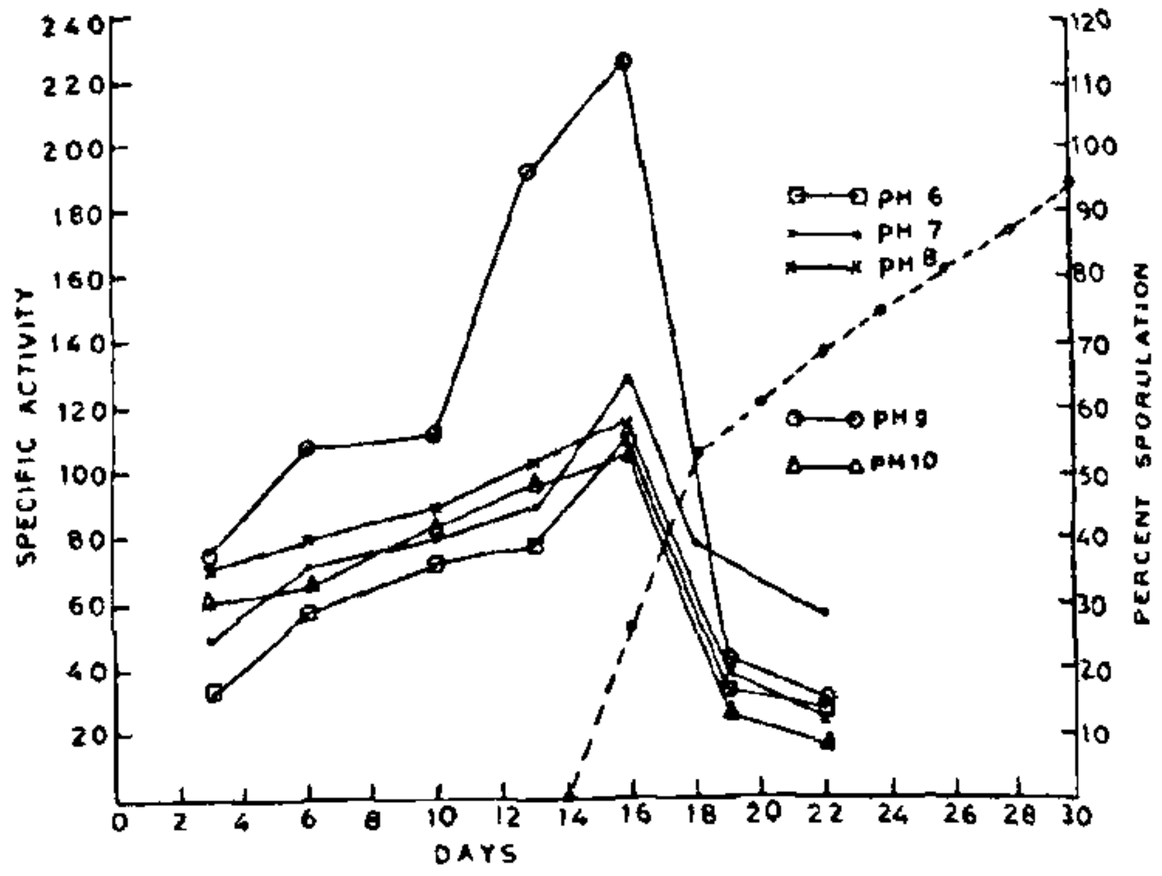

Figure 1. pH activity profile of protease enzyme in *Anabaena* spores and percentage of sporulation (o---o---o)

Table 1 *Biochemical metabolite changes in Anabaena sp spores during germination and sporulation percentages**

Metabolites	14th day	16th day	18th day
Chlorophyll-a (mg/g†)	0.138 (0)	0.162 (26.04%)	0.104 (52%)
Carotenoids (mg/100 mg†)	0.003	0.004	0.002
Carbohydrates (mg/100 mg†)	0.290	0.400	0.250
Proteins (mg/100 mg†)	0.053	0.060	0.034

* sporulation percentage is in parenthesis; † Fresh weight of alga

(b) *Effect of pH on the enzyme activity*: The activity of the enzyme was studied with casein as substrate at different pH values. It is clear from figure 2 that the protease activity was maximum at pH 7 and 9. This indicates the presence of neutral and alkaline range proteases in the spores of this alga.

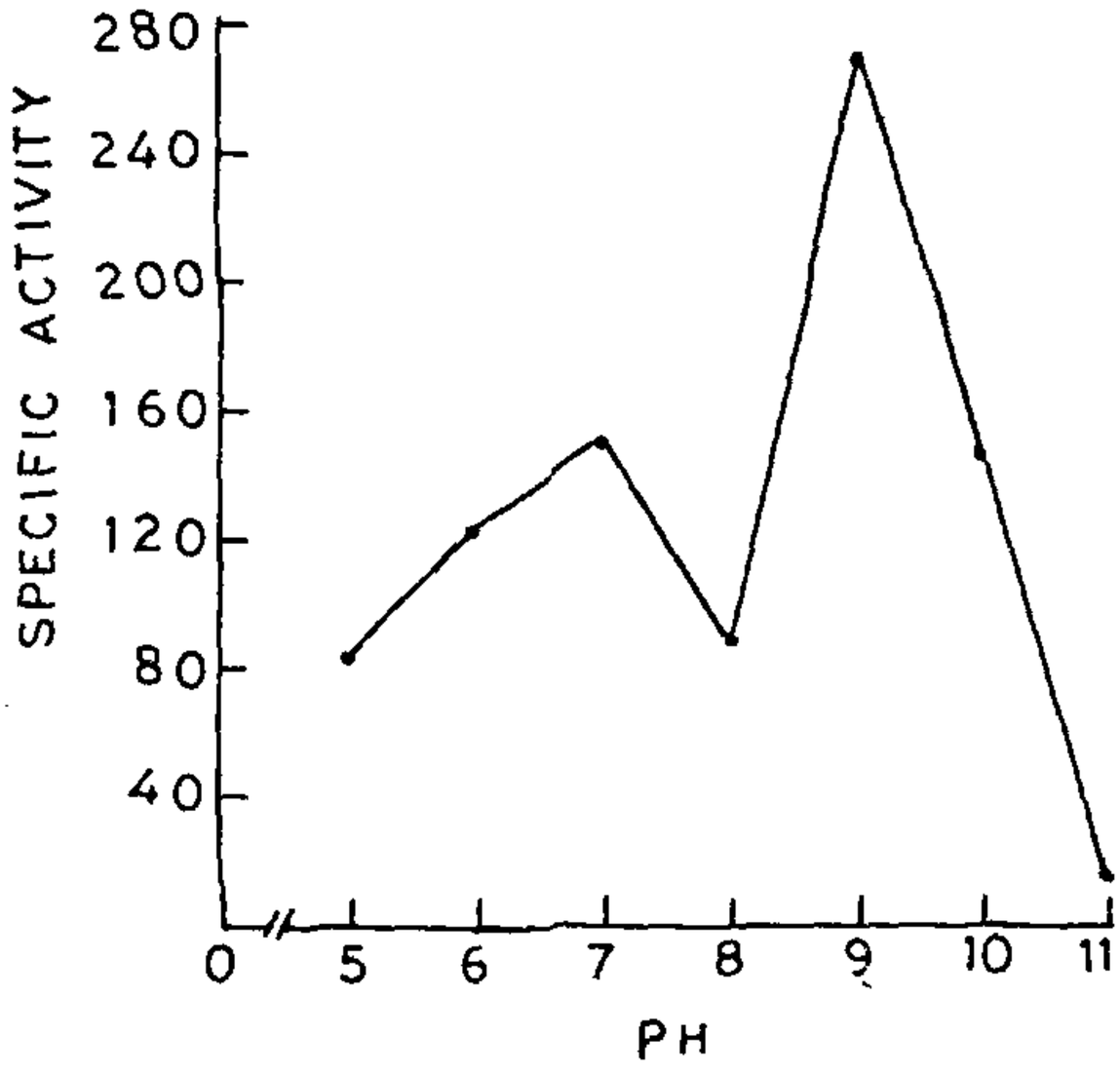

Figure 2. Protease enzyme activity at different pH.

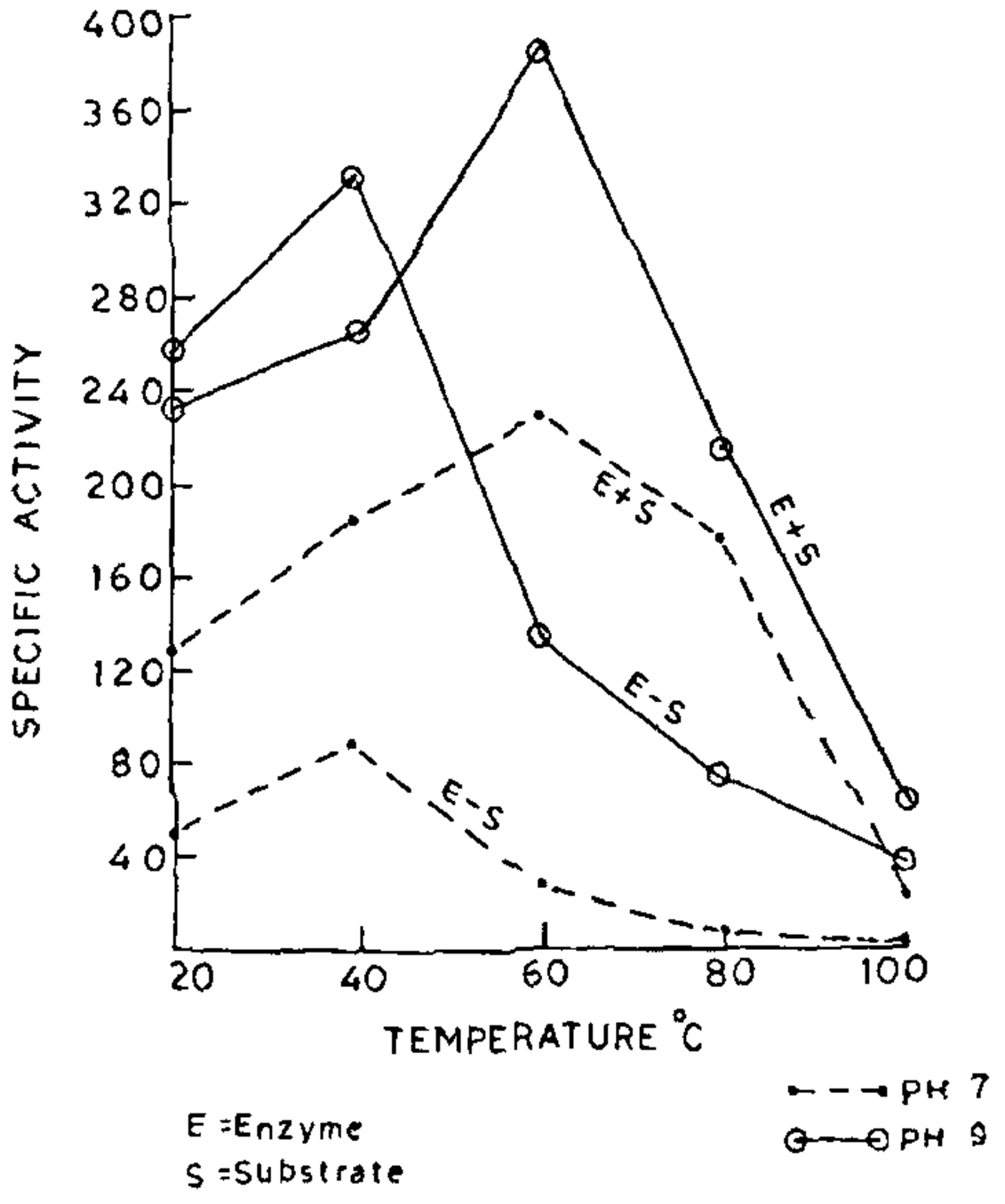

Figure 3. Effect of temperature on protease activity.

(c) *Effect of temperature on the activity of enzyme*: Protease activity was measured at different temperatures at pH 7 and 9. The stability of enzyme at different temperatures was investigated by taking only aliquots of the enzyme without substrate in separate tubes and preincubating them for 10 min at temperatures ranging from 20°C to 100°C and then assaying for protease activity. It is evident from figure 3 that the activity was stable upto 40°C and above this temperature it gets inactivated at pH 7 as well as pH 9.

When the enzyme extract was incubated with an equal volume of the substrate at different temperatures (20, 40, 60, 80 and 100°C) for 10 min, the enzyme exhibited rather optimum at 60°C. It may be due to the fact that the denaturation of the enzyme is prevented in the presence of the substrate. The results are similar to the observations made in germinating seeds of bajra[3].

(d) *Effect of metal ions and urea on the enzyme activity*: Protease activity was assayed in the presence of different divalent metal ions and urea at the final concentration of 5 mM. The results showed that Ca^{++} and Hg^{++} inhibited the protease activity at pH 7 and 9. Mg^{++} and Cu^{++} stimulated the enzyme activity at these pH values. Protease activity was not reduced when the enzyme was assayed in the presence of metal chelate, EDTA. It appears that the metal ions are not involved in the protease activity. The enzyme activity was inhibited by urea (5 mM) at pH 7 and 9 (table 2).

Table 2 *Effect of metal ions and urea on the enzyme activity (final concentration of the individual metal ions in the assay mixture was 5 nM)*

Addition	Protease activity (%of control) pH 7	pH 9
None (control)	100.0	100.0
$CaCl_2$	45.45	62.5
$HgCl_2$	50.1	63.8
$MgCl_2$	150.1	194.1
$CuSO_4$	149.3	198.4
Urea	22.7	73.3

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NEWS

SORTING OUT SCIENCE AND PSEUDOSCIENCE

. . . "Scientists and philosophers tend to treat superstition, pseudoscience, and even antiscience as harmless rubbish, or even as proper for mass consumption; they are far too busy with their own research to bother about such nonsense. This attitude is most unfortunate for the following reasons. First, superstition, pseudoscience, and antiscience are not rubbish that can be recycled into something useful; they are intellectual viruses that can attack anybody, layman or scientist, to the point of sickening an entire culture and turning it against scientific research. Second, the emergence and diffusion of superstition, pseudoscience, and antiscience are important psychosocial phenomena worth being investigated scientifically and perhaps even used as indicators of the state of health of a culture. Third, pseudoscience and antiscience are good test cases for any philosophy of science. Indeed, the worth of such philosophy can be gauged by its sensitivity to the differences between science and nonscience, high-grade and low-grade science, and living and dead science."

[Mario Bunge (McGill U., Montreal) in *Skeptical Inquirer* 9(1): 36–46, Fall 84. Reproduced with permission from Press Digest, *Current Contents*®, No. 3, January 21, 1985, p. 12. (Published by the Institute for Scientific Information®, Philadelphia, PA, USA.)]

STORAGE POWER PLANTS

A hydropower storage plant now in the making near Leningrad will consume at night energy from thermal and nuclear power plants in the north-western USSR. During the peak hours, it will return to the power grid. The plant, which will be located on a high hill by the Shapsha River, will have eight power units of 220,000 kilowatts each. At night its recuperative generators will pump water back into a reservoir on the hilltop to be used for electricity generation during the peak hours.

The new storage plant will help get more electricity from the operating thermal and nuclear power stations, whose power units are run at a slower pace at night when electricity is not consumed, burning more fuel per kilowatt-hour produced. The new storage plant will generate more than 1,500 million kilowatt-hours annually. (*Soviet Features*, Vol. XXIV, No. 34, Information Department of the USSR Embassy in India, No. 25, Barakhamba Road, New Delhi 110 001)