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Heterogeneity of the acid phosphatase and ribonuclease from protocorms of the orchids *Cymbidium* Sw. and changes occurring after treatment with streptomycin

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Abstract

The molecular forms of the acid phosphatase and RNase in protocorms of *Cymbidium* Sw. were studied by disc electrophoresis. The effect of streptomycin added to the culture medium on both enzymes was investigated. Significant changes in enzyme activity and electrophoretic patterns occured after addition of streptomycin at the beginning of culture growth. This indicates that the enzymes are affected by streptomycin in early stages of development of the protocorms.

INTRODUCTION

Studies on isoenzymes of higher plants are commonly related with observations on changes in enzyme patterns occuring during seed germination and ripening as well as during growth and development of the plants. The presence of multiple enzyme forms in tissues is usually demonstrated by separation of proteins in gel electrophoresis.

Maravolo et al. (1967) observed changes in the pattern of acid phosphatase during sexual development of *Marchantia polymorpha*. Keswani and Upadhya (1969) showed that during seed germination of *Saguara cactus* the ammount of the acid phosphatase activity zones was gradually increased and the full isoenzyme set was completed between 71 and 96 hours of germination. In apple seeds 10 molecular forms of acid phosphatase were observed and during their development changes in the relative activity of these forms were stated (Rychter and Lewak, 1969). In further studies it was shown that the activity of this enzyme was controlled by plant hormones, giberellin and

abscisic acid (Rychter et al., 1971). Rudolf and Stahman (1966) observed that in bean leaves infected with *Pseudomonas phaseolica* changes in the intensity of the acid phosphatase activity bands occured which were dependent on the infection period.

Many studies performed on molecular forms of the acid phosphatase and other enzymes in plants showed that not all molecular forms are synthetized at the same time but some of them appear later, after the plant has reached a proper developmental stage.

Protocorms of orchids which are early developmental stages of the plant are characterized by intense biosynthesis processes. They are thus useful objects to observe changes occuring in molecular forms of enzymes during growth and differentiation and to study the effect of substances inhibiting protein biosynthesis. Thus the aim of this work was to observe the behaviour of molecular forms of acid phosphatase and ribonuclease during development of orchid protocorms of the genus *Cymbidium* Sw. and also the effect of streptomycin on both enzyme activities.

MATERIALS AND METHODS

Studies were performed on protocorms of *Cymbidium*, clone Lib. 66/9 obtained from meristematic tissues in the Botanical Garden in Wrocław. The protocorms were cultivated in sterile conditions, in a liquid medium after Tsuchiy as modified by Kukułczanka (1970). The technique of protocorm cultivation is described in detail by Kukułczanka (1970) and Kukułczanka and Paluch (1971).

Parallelly to control cultures protocorms were also grown in the presence of streptomycin added to the medium in ammounts of 5 or 10 mg per 1 l of the medium. The antibiotic has been added either at the beginning of culture growth or after a 3 weeks' growth period. The cultures were grown for 9 or 17 weeks.

Preparation of extracts from orchid protocorms

Agglomerates from 9 or 17 weeks old protocorms were washed several times with distilled water, then dried on filter paper, weighed and afterwards homogenized in a glass Potter's homogenizer with 0,9% Nacl, 0.1 M acetic buffer, pH 5.0 or with distilled water. 2 ml of the extracting solution were used per 4 g of tissue. The homogenates were centrifuged at 16.000 r.p.m. at 4° for 15 minutes. In the clear supernatants protein and acid phosphatase as well as RNase activities were determined; then the extracts were subjected to electrophoresis on polyacrylamide gels.

Analytical methods

Protein in the extracts was determined by the turbidimetric tannin micromethod after Mejbaum-Katzenellenbogen (1955). The acid phosphatase activity was estimated with sodium phenylphosphate as substrate at 37°, pH 5.0 by measuring the amount of liberated inorganic phosphate after 30 minutes by the method of Fiske-Subba-Row. The specific activity of acid phosphatase was defined as the amount of μg of inorganic phosphate liberated in 1 minute per mg of protein at 37°, pH 5.0. Ribonuclease activity was measured after Anfinsen (1954). The extinctions were read on a Zeiss VS-W1 spectrophotometer in 1 cm cells at 260 nm.

Electrophoresis on polyacrylamide gels

Electrophoresis was carried out in 0.5 × 7.0 cm glass tubes according to Ornstein (1964) and Davis (1964) at pH 9.5 using 7.5% gels with 0.0005% riboflavin as initiator of photopolymerisation. The concentrating gel contained 20% sacharose. About 0.1 ml of the extract containing from 80 to 100 μg protein dissolved in 20% sacharose was applied on the surface of the gel. Electrophoresis was carried out at 4°C for 1.5 to 2 hr at 2.5 mA per tube for the first 15 minutes and continued with 4 mA per tube. After electrophoresis the gels were incubated twice for 20 minutes in 0.2 M acetic buffer, pH 5.0. Acid phosphatase activity was localized on the gels by the diazo coupling technique using sodium alpha-naphtyl phosphate and Fast Garnet GBC (1 mg of each per 1 ml of 0.2 M acetic buffer, pH 5.0). Ribonuclease activity was detected on the gels by the method of Wolf (1968) and also after Curtis and Wilson (1969). The latter method allows also for quantitative estimation of RNase activity eluated from the gels. In both methods RNase activity was visualized in form of destained bands on a dark blue background.

RESULTS

Initial experiments showed that the average amount of protein extracted from protocorms by distilled water, 0.9% NaCl and 0.1 M acetic buffer, pH 5.0 amounted to 1 mg per 1 ml of the extract. The specific activity of acid phosphatase was about 22 units. In the case of ribonuclease however, the highest activity was observed in acetic buffer extracts. On the base of these results further experiments were performed on proteins extracted with 0.1 M acetic buffer, pH 5.0.

Acid phosphatases and ribonucleases of control cultures of the orchid protocorms

In Fig. 1A a typical zymogram of the acid phosphatase obtained from acetic buffer extracts from 17 weeks old orchid protocorms is shown. After polyacrylamide gel electrophoresis the acid phosphatase activity was localized in 3 zones. In each zone two electrophoretically

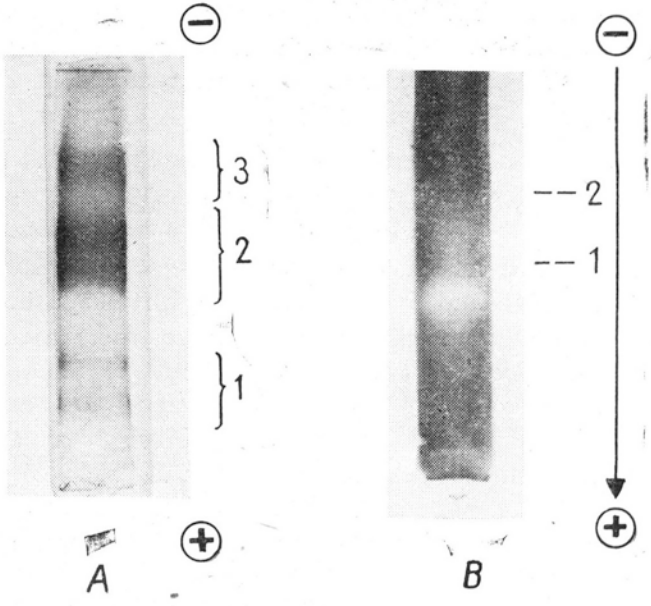

Fig. 1. Zymograms of the acid phosphatase and ribonuclease from acetate extracts of protocorms of *Cymbidium* Sw.

A — zymogram of acid phosphatase; *B* — Zymogram of ribonuclease.

A — Acid phosphatase activity on the gels was detected with alfa-naphtyl acetate and Fast Blue B. 80 ug of proteins of the acetate buffer extracts were subjected to electrophoresis; *B* — Ribonuclease activity on the gels was detected after Wolf (1968). 100 μg of proteins of the acetate buffer extract were subjected to electrophoresis. Electrophoresis was carried out on 7.5% gels at pH 9.5 after Davis (1964). Details under Methods.

distinct bands can be distinguished. The enzyme pattern thus suggests the existence of three molecular forms of the acid phosphatase with additional microheterogeneity. The lowest acid phosphatase activity appeared in the zone showing highest anodical mobility. Zymogram of the ribonuclease is shown in Fig. 1B. The pattern shows two closely separated zones of ribonuclease activity differing markedly in intensity. The faster migrating ribonuclease is the predominating form of the enzyme.

Studies performed on 9 or 17 weeks old protocorms showed that there were no significant differences in the number and intensity of the electrophoretically separated bands in both studied enzymes.

The effect of streptomycin on acid phosphatase and ribonuclease activities during growth of protocorm cultures of orchids

Streptomycin was added to the liquid culture medium in two stages: at the beginning of the culture growth — experiment I, and after 3 weeks growth of the culture — experiment II.

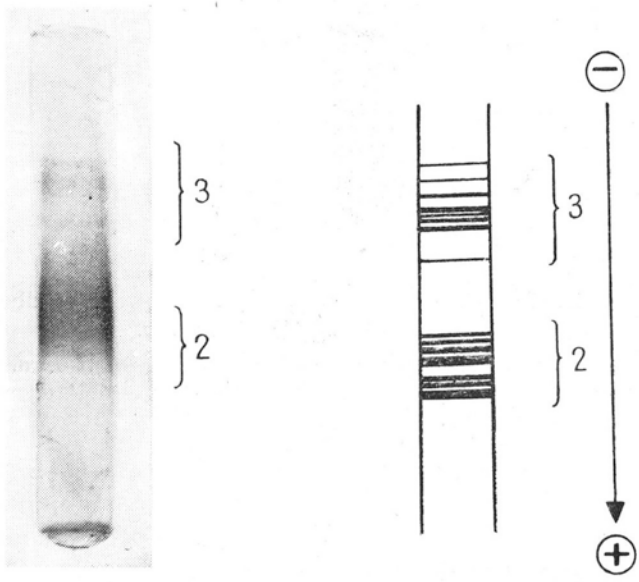

Fig. 2. Effect of streptomycin on the electrophoretic pattern of acid phosphatase from protocorms of *Cymbidium* Sw.

Streptomycin was added to the medium at the beginning of culture growth. After 7 weeks of growth acetate buffer extracts from protocorms were made and 80 μg of proteins were subjected to disc electrophoresis.
Staining for enzyme activity. Details under Methods.

Experiment I. The addition of streptomycin to the medium at the beginning of culture growth caused strong inhibition of protocorm growth, their green colour, in contrast to the untreated protocorms, was much lighter. In Fig. 2 zymogram of the acid phosphatase of protocorms cultivated in the presence of streptomycin is shown. The fastest migrating molecular form disappeared and additional subbands were visualized in the region of slower migrating forms when this pattern was compared with that obtained from the untreated protocorms (Fig. 1 A). Similar patterns were obtained in all experiments independently on the added streptomycin dose. However, in this experiment no ribonuclease activity was detected in the gels, with both doses of streptomycin.

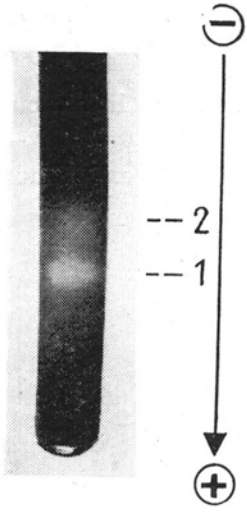

Fig. 3. Effect of streptomycin on the electrophoretic pattern of ribonuclease from protocorms of *Cymbidium* Sw.

Streptomycin was added to the medium after 3 weeks' growth of the culture. After 7 weeks acetate buffer extracts from protocorms were made and 100 μg of proteins were subjected to disc electrophoresis. Gels were stained for RNase activity. Details under Methods.

Experiment II. Protocorms treated with streptomycin after a 3 weeks growth period growed green and developed only slightly weaker than protocorms of the untreated cultures. In zymograms of the acid phosphatase no distinct differences were observed in the amount of bands as well as in their relative distribution, which, at both dose levels of streptomycin, were similar to that observed in control cultures. In this experiment the ribonuclease activity has not been completely inhibited, but differences were observed in the relative activity of the two bands, particularly in the faster migrating RNase (Fig. 3). To demonstrate the effect of the dose of streptomycin added to the medium on ribonuclease activity, quantitative determinations of the enzyme activity on gel slices after electrophoretic separation were performed according to Curtis and Wilson (1969). By the use of this method only one RNase activity band could be visualized, namely the faster migrating toward the cathode and predominating RNase. Quantitative estimations of enzyme activity in this fraction are presented in Tab 1. It has been shown that the addition of 5 mg of streptomycin per 1 l culture medium resulted in a 30% decrease of RNase activity and a twofold higher dose of this antibiotic caused a 50% loss of enzyme activity in comparison to the activity estimated in protocorms of untreated cultures.

DISCUSSION

Cultivation of protocorms in sterile conditions enables to obtain experimental material free of viruses and bacteria, this being of significant importance in observing changes occuring in isoenzyme patterns. It has been shown in this paper that the ribonuclease appeared in protocorms of orchids (*Cymbidium* Sw.) in form of two activity bands (at pH 5.0) only when the method of Wolf (1968) has been used to visualize the activity. Using the method of Curtis and Wilson (1969) however, which differs from the former one in a shorter incubation period with the substrate and in the dye used to stain the undigested RNA, only the main RNase activity zone has been detected. The method of Wolf (1968), although in our case more sensitive, allows only for qualitative detection of the enzyme whereas using the method of Curtis and Wilson (1969) also quantitative estimations of the RNase activity eluted from gels after electrophoresis can be performed. By the use of this method we were able to demonstrate the effect of the dose level of streptomycin on enzyme activity.

Table 1

Effect of streptomycin on the fast moving RNase in *Cymbidium* Sw.

Dose of streptomycin mg/l medium	Protein concentration, μg/ml	E_{260}	Specific activity E/mg protein
0	80	0.105	1.3
5	66	0.055	0.803
10	65	0.040	0.615

Results are summarized from 3 independent experiments. RNase activity in gels was estimated after Curtis and Wilson (1969). After electrophoresis, in one part of the gels the RNase activity was localized, from others the enzyme was eluted. In each experiment the enzyme was eluted from 3 parallel gel secetions. Extracts from parts of the unstained gels where no RNase activity was detected in the stained gels, served as controls. In the gel eluates the content of protein was estimated and the extinction was measured at 260 nm.

An interesting observation in this paper was to demonstrate the effect of streptomycin on acid phosphatase and RNase. When streptomycin was added to the culture medium at the beginning of culture growth no RNase activity was detected on the gels after electrophoresis whereas the acid phosphatase showed significant molecular changes which were reflected in the appearance of additional subbands in the main activity zones. When streptomycin was added to the medium after a three weeks' growth period of the protocorms only partial inhibition of ribonuclease activity could be observed and the acid phosphatase showed no significant changes in the enzyme pattern. It was thus shown that both enzymes are markedly influenced by streptomycin only in very early stages of development of *Cymbidium* Sw.

SUMMARY

The molecular forms of acid phosphatase and RNase of acetate buffer extracts (0,1 M, pH 5.0) from protocorms of the orchids *Cymbidium* Sw. were studied by disc electrophoresis. The acid phosphatase activity has been localized in three zones, each containing 2 activity bands. The RNase activity appeared in 2 zones differing markedly in intensity.

The effect of streptomycin added to the culture medium at different stages of growth, was investigated on both enzymes. No RNase activity after electrophoresis was detected on the gels, when streptomycin has been added to the medium at the beginning of the culture growth. The acid phosphatase, however, showed significant molecular changes which where reflected in the appearance of additional subbands in the main activity zones. When streptomycin was added to the medium after three weeks' growth of the protocorms, only partial inhibition of the RNase activity was observed mainly in the faster moving zone, whereas no changes were observed in the electrophoretic pattern of the acid phosphatase.

These experiments indicate that both enzymes are markedly influenced by streptomycin in very early stages of development of the protocorms of *Cymbidium* Sw.

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Heterogenność kwaśnej fosfatazy i rybonukleazy protokormów storczyków Cymbidium Sw. oraz zmiany zachodzące pod wpływem streptomycyny

Streszczenie

Heterogenność kwaśnej fosfatazy i rybonukleazy u protokormów storczyków z rodzaju *Cymbidium* Sw. badano za pomocą elektroforezy dyskowej. Aktywność kwaśnej fosfatazy zlokalizowano na żelach w 3 głównych strefach, a RNazę w dwóch blisko siebie leżących pasmach.

Badano wpływ streptomycyny na zachowanie się obu aktywności enzymatycznych we wczesnych stadiach rozwojowych protokormów storczyków. Streptomycyna dodana do hodowli w momencie jej zakładania hamowała rozwój protokormów oraz aktywność RNazową, natomiast kwaśna fosfataza uległa znacznym modyfikacjom, które odzwierciedliły się w wystąpieniu dodatkowej mikroheterogenności w obrębie głównych stref aktywności enzymatycznej. Streptomycyna dodana do hodowli po trzech tygodniach od momentu jej założenia wywołała tylko częściowe zahamowanie aktywności rybonukleazowej, głównie szybciej wędrującej RNazy, natomiast kwaśna fosfataza nie wykazywała istotnych zmian w obrazie elektroforetycznym.

W badaniach tych wykazano, że streptomycyna wywiera istotny wpływ na badane enzymy we wczesnym okresie rozwoju protokormów *Cymbidium* Sw.