ACTA SOCIETATIS
BOTANICORUM POLONIAE
vol. XLI — nr 2
1972

Activity and localization of some hydrolytic enzymes during the development of *Iris pseudoacorus* endosperm*

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(Received: July 24, 1971)

Abstract

The changes in the activity of some hydrolytic enzymes during the development of *Iris pseudoacorus* endosperm were investigated using biochemical and cytochemical methods. In the early stages of development the chalazal pole shows a greater enzymatic activity than the micropylar pole. These differences decline as the seeds mature, and the activity of the studied enzymes becomes lower as the endosperm develops. Considerable activity of ß-galactosidase has been observed at the time of deposition of storage hemicelluloses in the cell walls of the endosperm.

Activity of the cytochemically detectable hydrolases is localised in granules up to 2μ in diameter. Cytochemical observations in the electron microscope indicate that the activity of acid phosphatase is associated with spherosomes.

INTRODUCTION

Few biochemical studies concerned the changes in the activity of hydrolytic enzymes during the development of seeds. In view of the role of nucleic acids in the morphogenesis and synthesis of proteins, only nucleases, in particular ribonuclease, have been considered (Ledoux et al., 1962; Ingle et al., 1965; Johri and Maheshwari, 1966; Kulka, 1969). These studies were carried out on the whole endosperm tissue, without any attempt to localize the enzymatic activity in particular cellular structures. The cytochemical methods offer such a possibility — both on the level of the light and electrone microscope.

A choice of materiał for our studies has been provided by our earlier results (Olszewska and Gabara, 1966) revealing a polar differentiation of *Iris pseudoacorus* endosperm, particularily obvious in the early

* This work was partially supported by Polish Academy of Sciences, VI Department, Commision for Ultrastructure of Cell and Tissues.

stages of development. A more intense mitotic activity was found in the chalazal pole where in the nuclear endosperm the mitotic gradient was initiated and remained in the early stages in the cellular endosperm. In the early stages of development the chalazal endosperm was particularily rich in the endoplasmic reticulum, and in its external zone a considerable activity of the acid phosphatase had been cytochemically stated (Mikulska et al. 1967). Differences in the activity of the acid phosphatae and of β-galactoside occuring in the chalazal and micropylar poles of the endosperm had been earlier reported (Olszewska et al. 1968).

In the present paper we are demonstrating that the distinct differences in activity of some hydrolytic enzymes between the chalazal and micropylar poles in the early developmental stages of *Iris pseudoacorus* endosperm gradually disappear as the seeds mature. In mature seeds the endosperm presents the same enzymatic activity at both poles. The activity detectable by cytochemical methods is localized in cytoplasmic granules.

MATERIAL and METHODS

The endosperm of *Iris pseudoacorus* in the following developmental stages has been used:

I — seeds 4—4,5 mm long containing nuclear endosperm; their interior part is filled by a central vacuole; the embryo consists of under 20 cells;

II — seeds 5—6 mm long; the endosperm transforms from the nuclear to the cellular; the central vacuole is still present; the embryo consists of several dozens of cells;

III — seeds 7—8 mm long; the whole seed is filled with the endosperm tissue, which becomes white in colour; the embryo consists of a few hundred cells;

IV — seeds 8—9 mm long; the endosperm becomes yellow; the embryo is about 2 mm long;

V — ripe seeds; the endosperm is brown in colour; the embryo reaches the final dimension, which is about 4 mm in length.

In view of the small size of the embryo in stages I—IV, it was not removed from the material used for the biochemical studies.

1. Biochemical methods

Activity of acid β-glicerophosphatase (GPase), ribonuclease (RNase) and deoxyribonuclease (DNase) has been determined by the Holden and Pirie method (1955 a and b). Endosperm from each of the developmental stages mentioned above has been dissected out from the seed

coats and divided into two parts — the chalazal and the micropylar. About 500 mg of tissue have been used for analyses. The incubation has been performed at 30°C. Samples have been collected every 30, 60 and 120 minutes. The amount of inorganic phosphate released from particular substrates, tested colorimetrically by the method described by Holden and Pirie (1955 b) has been used for the estimation of the activity of the enzyme, which has been expressed in units of the enzyme needed for the release of 31 mg of P/1 during 1 hour.

As a substrate for GPase sodium β-glicerophosphate has been used, at the concentration of 60γ/ml in 0,25 citrate buffer at pH 5,0. As a substrate for the determination of the activity of RNase yeast RNA was used (previously dialized at low temperature) at the concentration of 400 mg P/l in a citrate buffer at pH 6,0. The DNA containing substrate at the concentration 400 mg P/l in a veronal buffer at pH 6,0 was used for the estimation of the DNase activity.

The experiments were repeated during three years (1966, 1967, 1968) with three repetitions at each collection.

2. Cytochemical methods

A. For the light microscope

Fragments of endosperm from each pole have been fixed in Baker's FoCa. The determination of enzymatic activity has been performed according to Pearse (1961). As a substrate for the determination of acid phosphatase α-naphtyl-phosphate has been used (Fast Blue RR salt, incubation 30 min. at 37°C). In order to determine the alcaline phosphatase, Naphtol AS-BI (Fast Red Violet salt, incubation 1 hr. at 37°C) and the method of Gomori were applied. Sites of non-specific esterase activity have been identified by means of α-naphtyl-acetate (Fast Blue RR salt, incubation 30 min. at 37°C), or by the Holt's indoxyl method (30 min. at 37°C). In Holt's method E600 as an inhibitor of esterases sensitive to organophosphate compounds was applied. β-galactosidase has been tested using as substrate 6-bromo-2-naphtyl-β-galactopyranoside (Fast Blue RR salt, incubation 2,5 hrs at 37°C).

In order to compare the quantitative changes in the activity of acid phosphatase and β-galactosidase the product of the reaction has been extracted with absolute ethanol for 18 hours at 60°C from the material in which the enzymatic reaction has been performed. The extinction of the extract has been measured photometrically in a Spekol photometer and its value has been related to the dry mass of the endosperm, out of which the coloured product of the enzymatic reaction has been extracted.

B. For the electron microscope

The chalazal pole of the endosperm in the I stage of development was fixed for 1 hour in 3% glutaraldehyde buffered to pH 7.2 with a cacodylate buffer and then washed three times in the buffer. Incubation for 45 minutes with sodium β-glicerophosphate according to modified method of Gomori without ammonium sulphide was followed by washing in the cacodylate buffer. The material was next placed for 2 hours into OsO_4. After dehydration the material was embedded in Epon 812. Ultrathin sections have been coloured in uranyl acetate and lead citrate for 15 minutes and examined in a TESLA electron microscope BS 512 A type with 80 kV.

RESULTS

In early stages of the development the chalazal part of the endosperm is characterized by high activity of the acid β-glicerophosphatase, ribonuclease and deoxyribonuclease. The activity of these enzymes is several times higher than in the micropylar part of the endosperm (Table 1).

Table 1

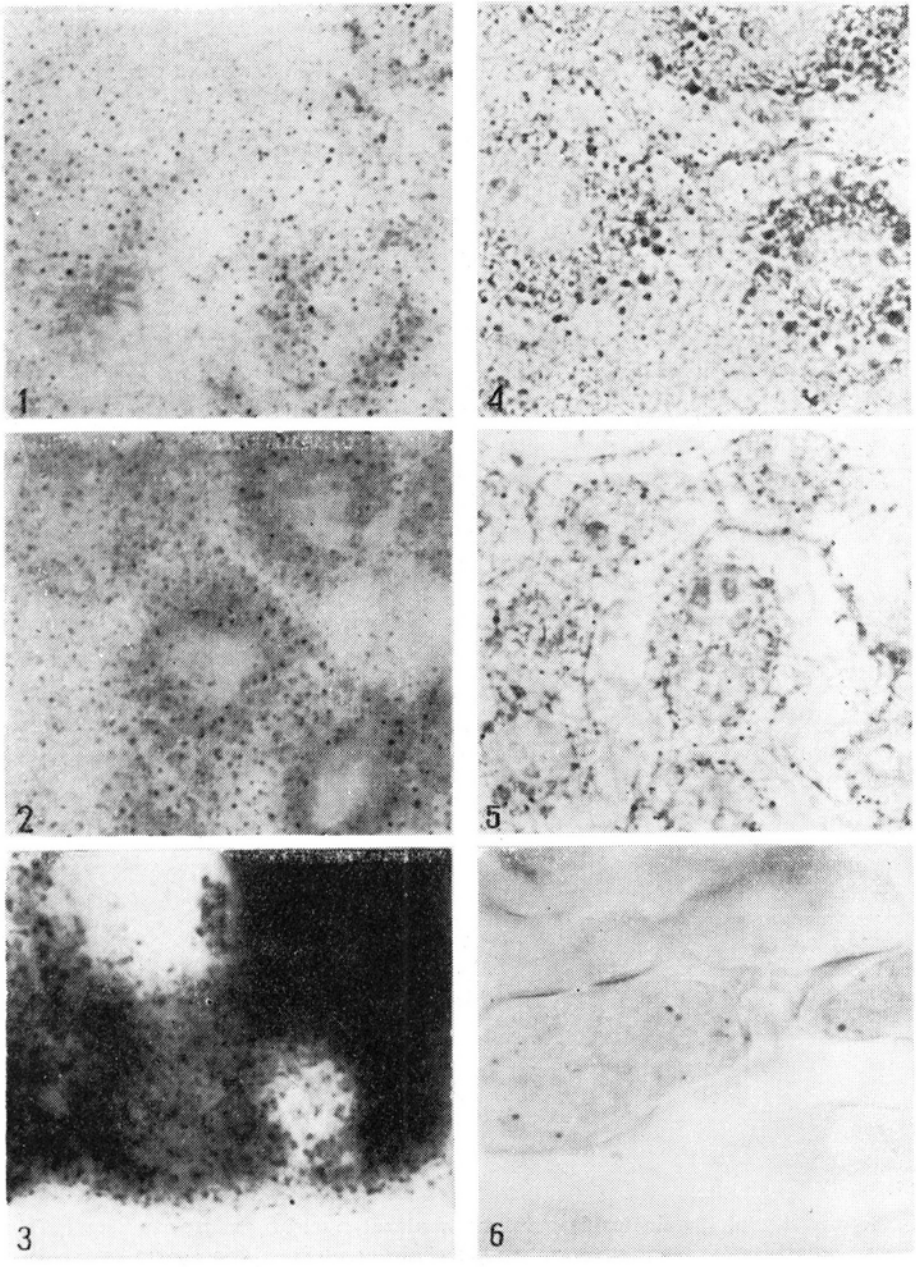
Activity of acid β-glicerophosphatase, ribonuclease and deoxyribonuclease in the endosperm of *Iris pseudoacorus* in the succesive stages of development, expressed in units per 1 g of fresh mass

Enzyme	Part of endosperm	Stage of development			
		II	III	IV	V
β-glicerophosphatase	chalazal	7.8	11.0	15.9	5.2
	micropylar	1.4	2.7	2.4	4.9
Ribonuclease	chalazal	12.2	12.3	5.6	3.1
	micropylar	1.8	2.6	0.8	2.9
Deoxyribonuclease	chalazal	4.1	4.1	2.1	1.0
	micropylar	0.6	0.8	0.2	0.9

The activity of the acid GPase in the chalazal pole achieves its maximum in the stage IV, and then declines. Though in the micropylar pole the activity is several times lower than in the chalazal part of endosperm, it increases with the maturing of the seeds so that it attains a level 3 times greater than in the stage when the central vacuole was still present. The activity of both nucleases examined is different: in the chalazal part it declines continously while in the micropylar part such a regularity was not observed.

The cytochemical results confirm the data obtained by the biochemical methods. All the hydrolases tested, i.e. acid and alcaline phosphatases, non-specific esterases, E600 resistant esterase and β-galactosi-

Plate I



Activity of acid phosphatase (substrate-α-naphtyl-phosphate, Fast Blue RR salt) in various developmental stages of *Iris pseudoacorus* endosperm; magnification ca 1000 x; 1, 2, 3 — nuclear endosperm from the same seed 4.5 mm long (stage I), simultaneously incubated

Fig. 1. Micropylar endosperm
Fig. 2. Subcentral endosperm
Fig. 3. Chalazal endosperm
Fig. 4. Subcentral cellular endosperm from stage II
Fig. 5. Subcentral cellular endosperm from stage III
Fig. 6. Chalazal endosperm from a mature seed (stage V)

Plate II

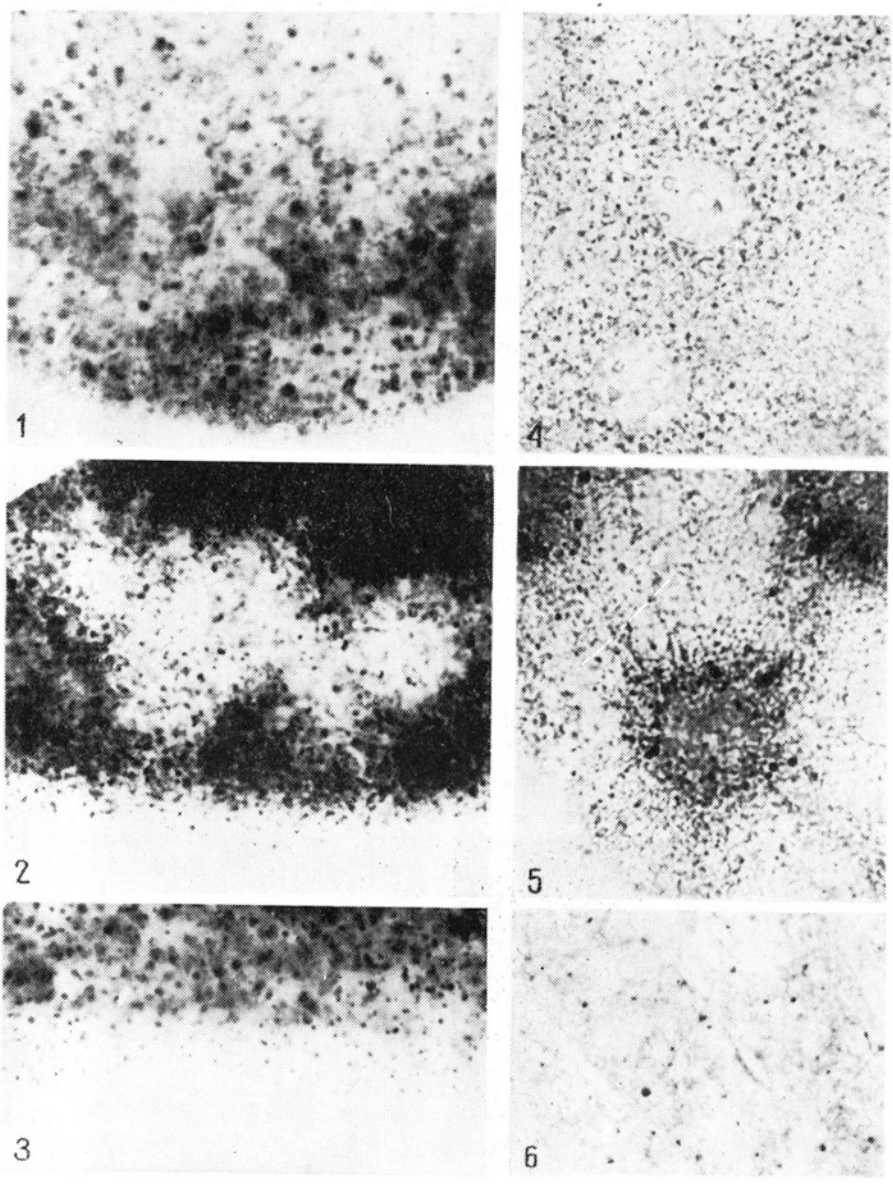


Fig. 1. Activity of β-galactosidase in the chalazal nuclear endosperm (stage I)
Fig. 2. Activity of indoxyl esterase in the chalazal nuclear endosperm (stage I)
Fig. 3. Activity of E600-resistant esterase in the chalazal nuclear endosperm (stage I)
Fig. 4. Activity of β-galactosidase in the micropylar nuclear endosperm (stage I)
Fig. 5. Activity of indoxyl esterase in the micropylar nuclear endosperm (stage I)
Fig. 6. Activity of indoxyl esterase in the micropylar cellular endosperm (stage II)

Magnification ca 1000 ×

Plate III

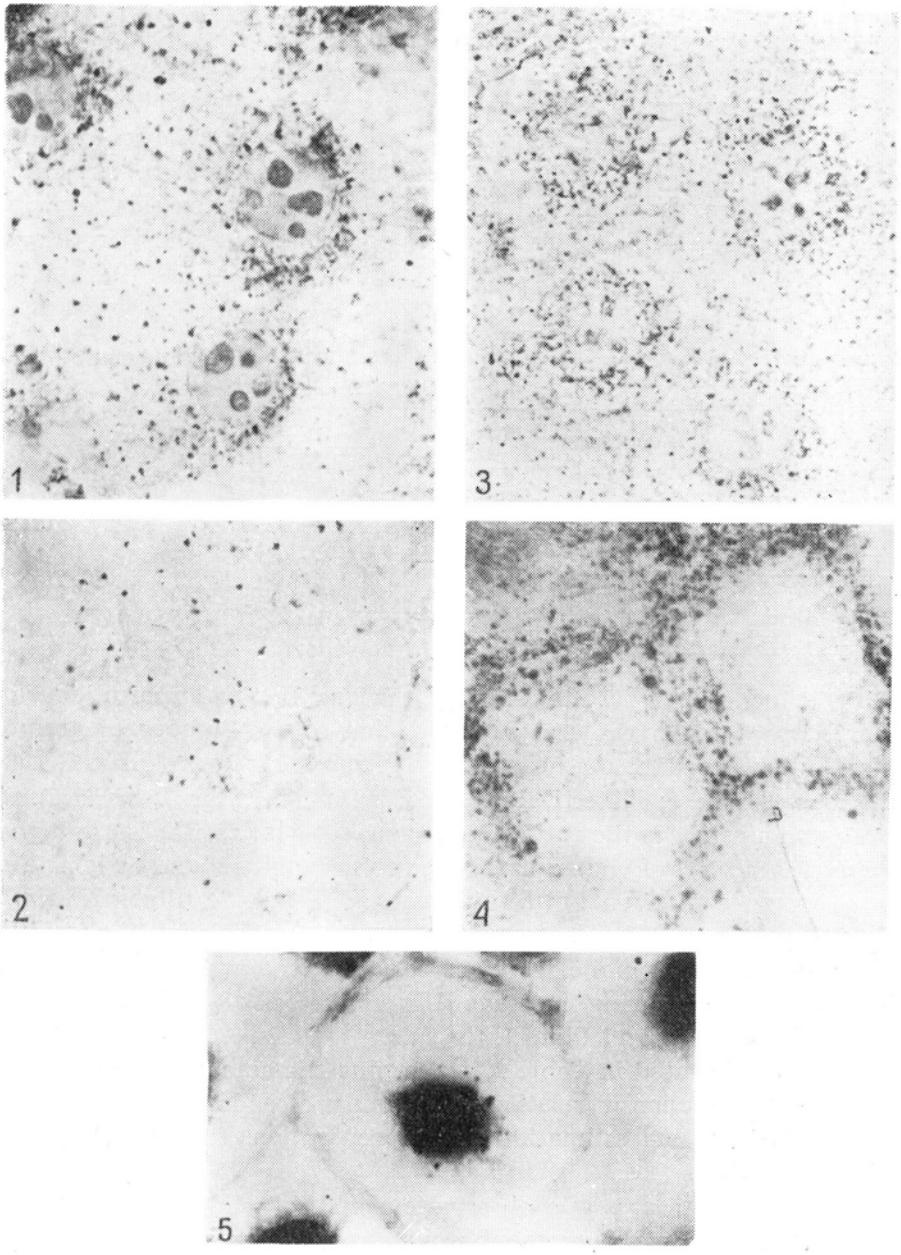


Fig. 1. Activity of alcaline phosphatase in the micropylar nuclear endosperm (stage I; substrate Naphtol As-BI, Red Violet LB salt)
Fig. 2. Activity of alcaline phosphatase in the micropylar cellular endosperm (stage III; substrate Naphtol AS-BI, Red violet LB salt)
Fig. 3. Activity of β-galactosidase in the micropylar cellular endosperm (stage II)
Fig. 4. Activity of β-galactosidase in the micropylar cellular endosperm (stage III)
Fig. 5. Activity of alcaline phosphatase in the micropylar cellular endosperm (stage III, Gomori's method)

Magnification ca 1000 ×

dase are most active in the chalazal pole during the I and II stage of endosperm development (Plate I, figs. 1-3, Plate II, figs. 1-5).

The reaction product revealing the activity of the examined enzymes is localized in the cytoplasmic granules, its size being between the limit of visibility of the light microscope to about 2 μ in diameter. In the case of indoxyl esterase the diffuse staining of the cytoplasm is visible. In the presence of E600 the indigo stains are to be found only in the granules (Plate II, figs. 2 and 3).

Table 2

Activity of acid phosphatase and β-galactosidase expressed in extinction per 1 g of *Iris pseudoacorus* endosperm dry mass

Enzyme	Part of endosperm	Stage of development		
		II	III	IV
Acid phosphatase	Chalazal	70.0	4.5	7
	Micropylar	62.0	4.1	5
β-galactosidase	Chalazal	50.5	65.5	16.5
	Micropylar	50.5	73.0	17.0

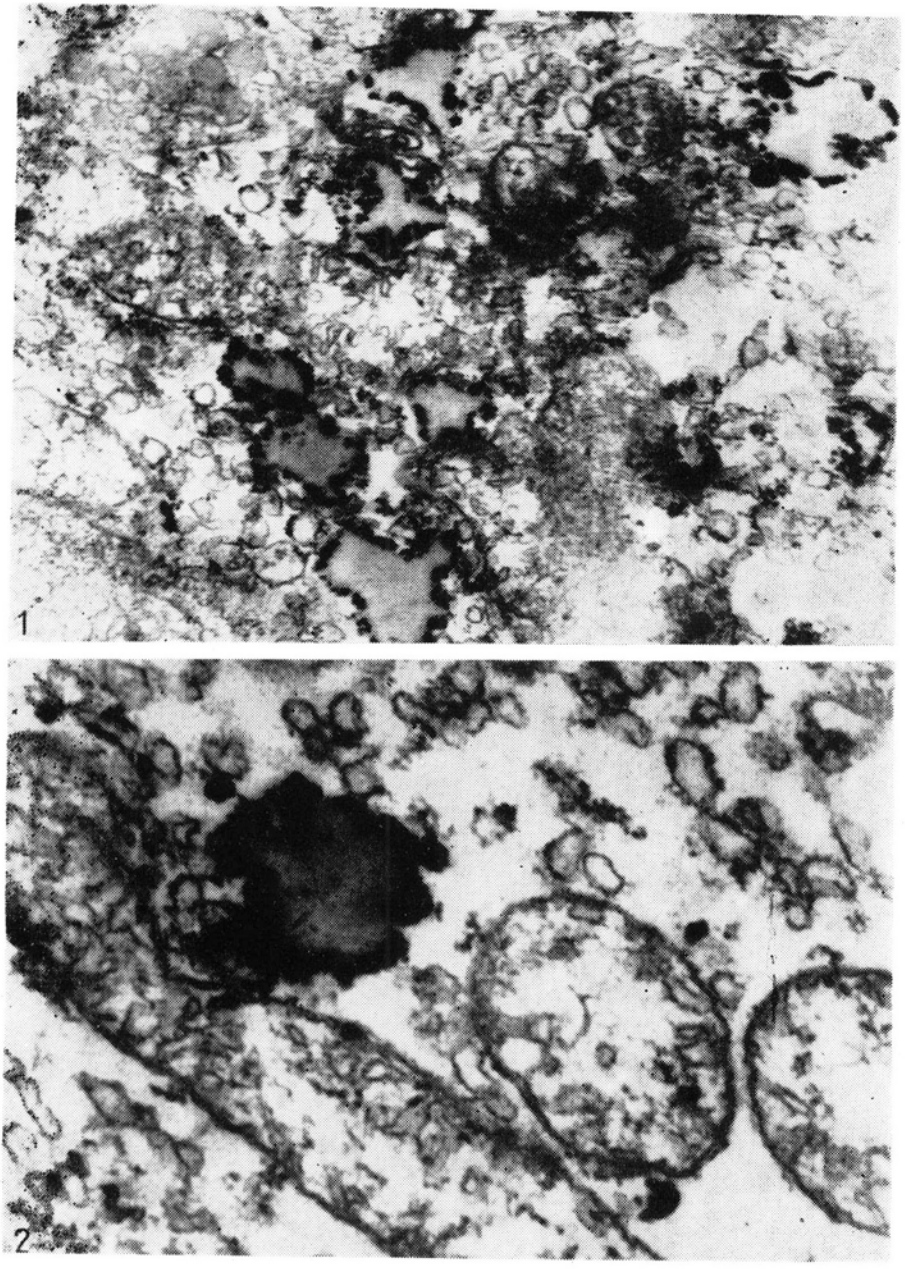
The cytochemical results indicate that as the endosperm develops the activity of the hydrolases declines, that is the number of granules containing the product of the reaction becomes reduced (Plate I, fig. 3 and 6, figs. 2,4 and 5; Plate II, figs. 5 and 6; Plate III, figs. 1, 2 and 5). Relatively long lasting is the activity of β-galactosidase, which is the highest during the stage III of endosperm development and is maintained at a relatively high level even until the stage IV (Table 2, Plate II, fig. 4; Plate III, figs. 4 and 5). Contrary to the other hydrolases, the granules with products of enzymatic reactions revealing the activity of β-galactosidase are localized during the stage III near the cell wall (Plate III, fig. 4; compare fig. 2 and 5 and Plate I, fig. 5).

The data presented in Table 2, concerning the amounts of products of the enzymatic reaction in relation to dry mass of endosperm are in agreement with the visual estimation of the preparations from various stages of development.

The electron miscroscope observations indicate in the chalazal pole the activity of an acid β-glicerophosphatase being associated exclusively with particles of an irregular shape, 0,2—0,5 μ in diameter. Structure of these particles is homogenous or fine-grained (Plate IV).

DISCUSSION

The use of both biochemical and cytochemical methods permits an evaluation of the quantitative changes in enzymatic activities and intracellular localization of the hydrolases tested.



Localization of the acid phosphatase activity in the chalazal nuclear endosperm (stage I) at the electron microscope level

Fig. 1. Magnification 40 200 ×; Fig. 2. Magnification 90 000 ×

In the endosperm of *Iris pseudoacorus* the nucleases activity (DNase and RNase) in the chalazal pole declines as the seed attains maturity, while in the micropylar pole the activity of these enzymes is several times lower and shows considerable fluctuations. A decline in the activity of nucleases in the endosperm of mature seeds has already been reported by several authors (Ingle at al., 1965; Johri and Maheshwari, 1966; Kulka 1969). Johri and Maheshwari (1966) have shown that the changes in the activity of RNase in the seeds of *Papaver somniferum* are associated with the changes in the RNA content. It was not possible to establish such a relation in the endosperm of *Iris pseudoacorus*, in which the differences in the content of DNA and RNA (in relation to the dry mass) was in both parts of the endosperm considerably lower than differences in the acitvity of nucleases (Konopska, in press).

The same level of the enzymatic activity in the two poles of the endosperm (in relation to dry mass) is achieved at the same time as the rapid growth of the embryo taking place between the stage IV and V.

It does not appear that changes in the activity of acid phosphatase and RNase have had any association with the mitotic activity in the endosperm of *Iris pseudoacorus*. Simola and Sopanen (1970) have indicated that in the isolated cells of *Acer pseudoplatanus* cultured in vitro, the highest activity of these enzymes occurs during the stationary phase, while the lowest — during the period of the greatest mitotic activity. In the endosperm of *Iris pseudoacorus* maximum of mitotic activity occurs in the early phase of development, i.e. during the stage in which the biochemically established RNase activity attains its maximum, as well as the visually estimated activity of the acid phosphatase.

Acid phosphatase and esterase participate in the digestive processes. Acid phosphatase and E600 resistant esterase are localized in the spherosomes of the epidermis of onion bulb (Wałek-Czernecka, 1965). In the endosperm of *Iris pseudoacorus* acid phosphatase was found in the globoids of storage proteins (Gabara and Modrzejewski, 1971). A considerable activity of the hydrolytic enzymes in the chalazal part of the endosperm of *Iris pseudoacorus* might be involved in the autolysis and desintegration of many cells in the marginal region of the chalazal endosperm (Mikulska et al. 1967). On the other hand, a considerable activity of acid phosphatase in sieve cells is considered as associated with the participation of this enzyme in the phosphorylation and dephosphorylation, and therefore in the transport process (Lester and Evert 1965). This role of acid phosphatase could be admitted in the chalazal part of endosperm which is the way of the transport of compounds for the developing seed.

The considerable activity and localization of β-galactosidase in the III stage of endosperm development should be emphasized. Granules containing products of the reaction revealing this enzyme are localized by

the cell walls when the storage hemicelluloses are being deposited. The storage hemicelluloses are the only form of storage polysaccharides in the endosperm of *Iris pseudoacorus* (Gabara and Modrzejewski, 1971). A similar localization of several glucosidases at the tip of a growing root hair and a simultaneous accumulation in this region of polysaccharides incorporating glucose ^3H has been established by Olszewska and Gabara (1967). Similar data, indicating an association between the activity of β-glactosidase and the metabolism of the cell wall, has been obtained by Keestra and Albersheim (1970). The glucosidases are known to catalyse not only the breakdown but also the formation of glycoside linkage, occuring in the synthesis of cell wall polysaccharides.

Analysis of the localization of activity of acid phosphatase at the electron miscroscope level indicates that the main carriers of this enzymes are the spherosomes. Both the dimensions and the shape of particles containing the deposits of lead, correspond to these structures. Similar results have been obtained by Mikulska and Gabara (1968) in the root meristem of *Allium cepa*. In the root meristem of *Cucumis sativus*, Poux (1970) has established the acid phosphatase activity not only in the bodies which could be spherosomes but also in small vacuoles. Such a localization of acid phosphatase was not observed in the endosperm of *Iris pseudoacorus*.

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Aktywność i lokalizacja niektórych enzymów hydrolitycznych w trakcie rozwoju bielma Iris pseudoacorus

Streszczenie

Metodami biochemicznymi i cytochemicznymi zbadano zmiany w aktywności niektórych enzymów hydrolitycznych w trakcie rozwoju bielma *Iris pseudoacorus*. W początkowych fazach rozwoju biegun chalazalny wykazuje większą aktywność enzymatyczną, niż biegun mikropylarny. Różnice te zacierają się w miarę dojrzewania nasion, przy czym aktywność badanych enzymów zmniejsza się w miarę rozwoju bielma. Znaczną aktywność wykazuje β-galaktozydaza w okresie deponowania na ścianach komórkowych bielma hemiceluloz zapasowych.

Aktywność wykrywalnych cytochemicznie hydrolaz zlokalizowana jest w ziarenkach o średnicy dochodzącej do 2μ. Wyniki badań cytochemicznych w mikroskopie elektronowym wykazują, że aktywność kwaśnej fosfatazy związana jest ze sferosomami.

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