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Research article

Activity of selected hydrolytic enzymes in *Allium sativum* L. anthers

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ABSTRACT

The aim of the study was to determine enzymatic activity in sterile *Allium sativum* anthers in the final stages of male gametophyte development (the stages of tetrads and free microspores). The analysed enzymes were shown to occur in the form of numerous isoforms. In the tetrad stage, esterase activity was predominant, which was manifested by the greater number of isoforms of the enzyme. In turn, in the microspore stage, higher numbers of isoforms of acid phosphatases and proteases were detected. The development of sterile pollen grains in garlic is associated with a high level of protease and acid phosphatase activity and lower level of esterase activities in the anther locule. Probably this is the first description of the enzymes activity (ACPH, EST, PRO) in the consecutives stages of cell wall formation which is considered to be one of the causes of male sterility in flowering plant.

1. Introduction

The pollen grain is structurally and physiologically equipped for being transferred onto the stigma of another plant. It is surrounded by a unique cell wall, i.e. the sporoderm composed of two layers: the outer exine and the inner intine. The intine of many plant species has been found to contain active cutinase, which is involved in stigma cuticle erosion, and pectin-degrading enzymes (Dearnaley and Daggard, 2001; Hiscock et al., 1994, Shayk and Kolattukudy, 1977). The intine is a storage site for esterolytic enzymes in *Olea* pollen, and it has been proved that these enzymes may be involved in germination and growth of the pollen tube and its penetration into the stigma (Rejón et al., 2012). In sterile *Leschenaultia formosa* pollen grains, in which the intine was not formed, no hydrolytic enzymes were detected (Knox and Friederich, 1971). The exine not only envelopes the pollen grain but is also involved in the first contact with the pollinated plant. The exine surface is richly sculptured and porous. With this structure, it can be a carrier of pollen-kit or tryphines, i.e. substances derived from the tapetum of the pollen donor. Therefore, the tapetum provides carbohydrates, lipids, pigments, proteins, and sporopollenin components on the surface of pollen grains. These substances fill the cavities in the exine sculpture and are the first to contact with the stigma surface, where they recognise their affinity through chemical receptors and are activated accordingly (Nettancourt, 2001). The mutual recognition between the stigma and pollen leads to cooperation of papillae in the hydration of pollen grains, which is indispensable for pollen tube germination. The germination process starts with release of substances synthesised by the cytoplasm of the pollen vegetative cell through the furrow or porus. The apex of the pollen tube covered by a very thin, flexible callose wall is the site of signal exchange. The growing pollen tube secretes hydrolytic enzymes: acid phosphatase, glucanases, and pectinases (Graaf et al., 2001, Lin et al., 1977). Not only the occurrence but also the distribution of many forms of esterases has been described in *Vicia faba* pollen. In germinating pollen and pollen tubes, a nonspecific esterase was detected in the cytoplasm and in the intine as well as a choline esterase located mainly in the sexine. A particularly large agglomeration of this enzyme was found in the porus (Bednarska, 1992). In pollen tubes of *Prunus avium* (cherry), acid phosphatase was localised in small vesicles and the ER system (Lin et al., 1977), while in *Crocus* pollen grains it was found in the intine (Knox and Heslop-Harrison, 1970). In the anthers of male-sterile mutants of *Zea*, the number of peroxidase isoenzymes was significantly higher than in the fertile form. However, the activities of acid phosphatases and esterases were substantially higher in fertile plants than in male-sterile mutants (Chaudhary and Minocha, 1989). These enzymes are assigned a role in hydrolysis of the middle lamellae between the stigmatic cells, which enable the apex of the pollen tube to penetrate the intercellular spaces easily towards the pistil transmitting tissue (Bosch et al., 2005).

Numerous experiments carried out with proteases and their

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inhibitors have shown that they regulate PCD *(Programmed Cell Death*) in plants by mutual control of their activities. Nystatin, i.e. a cysteine protease inhibitor and soybean trypsin inhibitor prevent cell death, and induced expression of cysteine or serine proteases or exogenous application thereof trigger cell death (Hatsugai et al., 2006). In *Arabidopsis thaliana* pollen, a CEP1 cysteine protease involved in tapetal PCD has been identified. In *cep1* mutants, interruption of tapetal PCD, abnormal development of the exine, and hence reduced pollen fertility were observed (Zhang et al., 2014).

The activity of the male gametophyte enzymes varies depending on the developmental stage and its physiological maturity. Therefore, the investigations were undertaken to determine the enzymatic activity of several enzymes in two different developmental stages: microspore tetrads and free mononuclear microspores liberated from a common callose wall. The study involved *Allium sativum* L., i.e. a sterile pollen-producing plant. The species is characterised by complete sterility, which prevents sexual reproduction of this species (Shemesh Mayer et al., 2013, Winiarczyk et al., 2012). The aim of this study was to conduct a qualitative and quantitative analysis of the activity of selected enzymes engaged in the formation of postmeiotic wall around the young pollen grain. Completely infertile *A. sativum* plants, were used as an experimental material. Two important developmental stages, namely tetrads and free mononuclear microspores were examined. Disturbances in developing of specific wall surrounding the mature pollen grains results in sterile pollen grain. A typical pollen wall (e.g. that of the model plant Arabidopsis thaliana) is mostly composed of sporopollenin, a complex and highly resistant biopolymer containing fatty acids, phenylpropanoids, phenolics and carotenoids, the intine is largely composed of pectin and cellulose (Albersheim et al., 2010). Mentioned above components for the construction of this wall are supplied from degenerating tapetum layer. In this process are involved in many enzymes including esterase, acid phosphatase and protease. These studies provide detailed information that furthers our understanding of various mechanisms in pollen wall development (Jiang et al., 2013).

2. Materials and methods

The plants analysed in this research originated from the Botanical Garden of Maria Curie-Skłodowska University. Anthers for the analyses were sampled from *A. sativum*, a plant that produces sterile pollen. Pollen was collected in two developmental stages: tetrads and mononuclear microspores. We collected flower buds of the determined length which correspond to established stages of development in the anthers. The correlations between the length of the flower bud and the development stage was previously examined by means of microscopic technics.

2.1. Preparation of the material for electrophoresis

A 75-mg anther sample was homogenised in an agate mortar with 400 μl of extraction buffer and the crude extract was shaken with water-dissolved phenol. Polypeptides from the phenol phase were precipitated with 0.1 M ammonium acetate in methanol for 24 h at a temperature of −18 °C. After centrifugation, the precipitate was dried under vacuum and dissolved in 25 μl of an aqueous solution containing urea, an ampholyte, and a detergent (Nonidet NP-40). The extraction procedure and the composition of the solutions applied were adopted after Hurkman and Tanaka (1986).

The two-dimensional electrophoresis was performed according to the procedure described by Hochstrasser et al. (1988). In the first dimension (protein electrofocusing in the isoelectric point – IEF), polypeptides were separated on polyacrylamide gels with Servalyte at pH3-10 polymerised in capillaries with a diameter of 0.8 mm and a length of 150 mm. The polymerised gel had a length of 140 mm, and the gel-devoid part of the capillary served as a chamber for transfer of the protein extract. After application of 12 μl of the protein extract at the cathode end, the polypeptides were separated for 18 h at a voltage increasing from 50 to 1000 V with the use of a stabilised power supply EPS 3500 (Amersham Pharmacia Biotech).

In the second dimension, the polypeptides were separated on 10% polyacrylamide plates (0,9 × 150 × 150 mm) polymerised without SDS. SDS was only present in the pH gradient buffer, in the gel columns, and electrode buffer. Before separation in the second dimension, the columns were immersed for 2 min in the pH gradient buffer. After the gel columns had been placed on the vertical polyacrylamide plates, polypeptides were separated at a constant current (15 mA/150 mm^2). After ca. 4 h of separation at room temperature, the polypeptides on polyacrylamide gels were stained with silver nitrate following the procedure specified by Heukeshoven and Dernick (1985). The gels with the stained polypeptides were scanned (SHARP JX-330) using the LabScan programme. The 2-DE images were processed in the Image Master 2-D Elite programme (version 3.1) from Amersham Pharmacia Biotech.

The isoelectric points and molecular weights of the separated peptides were determined by comparison with separated standards "Calibration Kit" (17-0582-01) supplied by Amersham Pharmacia Biotech, and the molecular weights were compared with the Molecular Weight Mixture M-5630 markers (SIGMA). For interpretation of the results of the isoenzymes, the intensity of enzyme staining was assessed using an LKB Ultrascan XL laser densitometer.

2.2. Enzyme extraction and separation

The principle of the procedure applied for enzyme separation was the same as that used for analysis of non-enzymatic polypeptides with slight modifications in the composition of the buffers used and some parameters of the two-dimensional electrophoresis method. In the second dimension, running gels containing 10% acrylamide and 50 mM Tris-glycine buffer, pH 8.9, containing 2 mM EDTA with SDS as a running buffer for each enzyme were used. Separation of polypeptides in the second dimension was conducted under 34 V for up to 22 h according to Kalinowski et al. (2002).

Protein determination was carried out according to instruction for GE Healthcare Life Sciences 2-D Quant Kit (Product code: 80-6483-56).

2.3. Specific activity of enzymes

The esterase activity was determined using *p*-nitrophenyl butyrate as a substrate (Hiscock et al., 1994). The hydrolytic activities of acid phosphatases and proteases were determined using *p*-nitrophenyl phosphate and labelled fluorescein isothiocyanate, respectively (Twining, 1984). Separations of esterase (EST), acid phosphatase (ACPH), and protease (PRO) were performed on 10% polyacrylamide gel in non-denaturating conditions, i.e. gel without SDS. The enzymes stained on the gels were analysed using the same programmes as for the protein image analysis and a laser densitometer was additionally used. The intensity of the stained isoforms reflected their activity (more intense staining = more active form).

Using the methods of electrophoretic separation, the activities of three hydrolytic enzymes, i.e. acid phosphatases, esterases, and proteases were compared in the extracts obtained from the *A. sativum* tetrads and released microspores. A unit of acid phosphatase activity corresponded to the release by the enzyme of 1 mM *p*-nitrophenol for 1 min at 37 °C. A unit of esterase activity corresponded to by the enzyme of 1 nmol of methanol from pectin for