Proteolytic enzymes from generative organs of flowering plants (Angiospermae)

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Abstract. Pollen proteases were discovered over 100 years ago, whereas the enzymes from female tissues have been used since the Roman era in simple biotechnological processes. In the last decade a great progress has been made in studies on plant proteases, including those from the generative organs. This paper reviews reports published in the last decade, concerning purification, properties and localization of proteases from generative parts of flowering plants against the background of the general proteolytic machinery of the plant. Special attention is paid to differences in protease structure and properties in comparison to other enzymes from the same catalytic classes. Participation of the proteases in all steps of pollen-pistil interaction as well as in pollen tube growth is discussed. Further intensive studies with use of native substrates are necessary to understand the role of proteases in pollination.

Key words: female tissue, pollen, pollen-pistil interaction, proteolytic enzymes.

Introduction

Proteolysis in plants, similarly as in other organisms, is not limited to simple degradation of proteins to be a source of amino acids through a recycling system, or going further, of carbon and nitrogen necessary for the synthesis of new polypeptides. Proteolytic events occur throughout the lifespan of the plant. Proteases control gene expression and are responsible for appropriate cell growth, differentiation, homeostasis, division or reproduction, as well as senescence. Proteolysis has an effect on processes like the supply of amino acids for synthesis of new proteins, control of the metabolism through the digestion of regulatory proteins and key enzymes, removal of damaged or improperly folded proteins, processing of proteins, zymogens and peptide hormones through digestion of signal peptides, and targeting of proteins to appropriate sites in the cell. Recently special attention has been paid to the role of the proteolytic complex in essential mechanisms, such as programmed cell death (PCD), protection against pathogens, control of the cell cycle, and signaling. Sometimes the processes mentioned above are closely related and occur at the same time and location. Selective proteolysis is directly or indirectly engaged in most processes in the plant cell (Callis 1995).

The gametophytic phase in the life of flowering plants is extremely reduced, as it is limited to the pollen grain (microgametophyte) and the ovule (macrogametophyte). Pollen grains, originating from pollen mother cells during meiosis, are released from anthers during pollen discharge. In spite of its simple two- or three-cell structure, the pollen grain participates actively in a series of complex processes on the surface of the stigma, such as adhesion, hydration and signal exchange with the female tissue in order to germinate into
a pollen tube. The pollen tube, penetrating the transmission tissue of the pistil, reaches the embryo sac, in which the ovule is fertilized (Bedinger 1992). All these mechanisms require participation of the enzymatic complex, including proteases (Heslop-Harrisson 1987; Mascarenhas 1990; de Graaf et al. 2001; Swanson et al. 2004). The presence of proteolytic enzymes in the pollen grain, the relatively readily available haploid material of flowering plants, was found as early as over 100 years ago (Green 1894). In turn the generative female tissue (most frequently in the form of dried flowers) containing protease was used in the Mediterranean for cheese production already during the Roman era (Heimgartner et al. 1990).

Degradation of proteins in the cytosol and nucleus is primarily under the control of ubiquitin/26S proteasome pathway (Sullivan et al. 2003; Vierstra 2003). Its importance for plants is indicated by the fact that in the genome of Arabidopsis thaliana over 1300 genes were identified (i.e. ~5%) for proteins connected with it. Besides, more than 600 of the identified protease genes may contribute to changes in the protein pool, essential for the cell (Schaller 2004). Protein degradation in plants involves also other proteolytic pathways functioning in different cell compartments (Vierstra 1996). Some of the enzymes are multi-subunit complexes requiring ATP to be active, as in the case of most of the chloroplast proteases (Schmidt et al. 1999; Zach and Clarke 2002). A specific kind of enzymes are intramembrane proteases, having several transmembrane domains (Urban and Freeman 2002). All these examples show that controlled proteolysis is a complicated process, engaging enzymes with different structures, specificities of action and roles.

Proteases are divided into several catalytic classes due to the presence of characteristic amino acids or metal ions in the active site. The most common are serine proteases, cysteine proteases, aspartyl enzymes and metalloproteinases. One of the examples of plant proteases belonging to threonine catalytic class are β-subunits of proteasome core. A novel class of proteases with glutamate as a catalytic residue has been identified recently. In some proteolytic enzymes the glutamate moiety activates the water molecule during the reaction of cleavage of a peptide chain (Fujinaga et al. 2004).

Considerable progress has been made in the last decade in studies on proteolytic plant enzymes and on processes in which they participate, as has been reported in numerous excellent review papers (Callis 1995; Vierstra 1996; Mutlu and Gal 1999; Callis and Vierstra 2000; Beers et al. 2000; Zach 2002; Estelle 2001; van der Hoorn and Jones 2004; Simoes and Faro 2004; Schaller 2004). However, they concerned either proteases from vegetative plant tissues or the role of proteolytic enzymes in one specific process. The aim of this paper is to collect and summarize reports, primarily from the last few years, concerning proteases from generative parts of flowering plants. Differences in their properties in comparison to enzymes belonging to the same catalytic classes are emphasized and their role during pollination is defined. Special attention is paid to pollen enzymes, which apart from their physiological functions in the pollen-pistil interaction are of special interest as one of the main causes of allergies.

**Proteases from female organs**

The ovules of flowering plants are difficult to obtain in the amounts sufficient for extraction and purification of proteolytic enzymes. For this reason studies have focused primarily on the characteristics of proteases of the ovary pistil style and stigma. Three proteases from cardoon (Cynara cardunculus) are the best characterized ones among those identified in female plant organs. The enzymes, isolated and purified from dry flowers, are called cyprosins, and previously were referred to as cynaras (Heimgartner et al. 1990). Each of them is a dimer with a larger subunit of 32–35 kDa, and a smaller of 13–16 kDa, forming as a result an enzyme of ~49 kDa. Inhibitory studies showed that they belong to the aspartyl class, with optimum action at pH 5.1 and are glycosylated by oligomers with high mannose contents. All the three proteases are specifically located in the cells of the epidermal layer in the style of mature flowers and their synthesis is under a strong tissue-specific regulation. Their functions have not been fully clarified, although their location and special accumulation in mature flowers seem to indicate their role in senescence. On the other hand, the significantly lower specific activity of cyprosins 1 and 2 in comparison to cyprosin 3 suggests that the two former enzymes may play a more subtle role in the processes occurring in the female tissue. Results of investigations indicate that the enzymes are encoded by three related genes. The cDNA sequence of cyprosins
codes for a chain of 473 amino acids, from which N-terminal prosequence is removed. During further processing, successive digestions of various peptide oligomers take place, giving in consequence three heterodimer forms (Cordeiro et al. 1994).

The next two proteolytic enzymes, cardosins A and B, were isolated and purified from fresh cardoon stigmas (Verrisimo et al. 1996). Both proteases turned out to be dimers composed of subunits of 31 and 15 kDa for cardosin A, and of 34 and 14 kDa for cardosin B. Inhibition studies showed that both enzymes belong to the aspartyl class, with optimum action at pH 5.0, i.e. similar to that of cyprosins 1–3 and relatively high in comparison to the other enzymes of that class (pH 2.0–4.0) in plants. Cardosin A is found in much larger amounts than cardosin B, and is less active but more specific in action. The amino acid sequence of cardosin is similar, but not identical to that of cyprosins described above. Probably cyprosin and cardosin genes have a common origin. Both cardosins differ in their catalytic properties and cardosin B does not yield any immunological cross-reactivity for the larger subunit of cardosin A. These results indicate that the two enzymes are products of separate genes, originating most probably as a result of duplication. The expression of cardosin A is limited to pistils and the enzyme itself is found mainly in vacuoles accumulating storage proteins of papillary layer cells of the stigma and in smaller amounts in the epidermis of the style. The possibility of transfer of the enzyme from vacuoles to cell walls and to the apoplast cannot be excluded (Ramalho-Santos et al. 1997). Moreover, it is involved in flower senescence and defense against pathogens. Cardosin A is the only plant enzyme of the aspartyl class containing the RGD sequence (Arg-Gly-Asp), the role of which will be mentioned while discussing the pollen-pistil interaction (Frazao et al. 1999). Cardosin B is found in large amounts in the cell wall and, as the first aspartyl enzyme, in the intercellular matrix of the transmission tissue of mature flowers. Due to the higher activity but low specificity of its action, cardosin B has been described as an enzyme non-specifically degrading the pool of proteins. However, its location indicates a protective action and involvement in yet undiscovered functions during the development of the pistil and the pollen-pistil interaction (Vieira et al. 2001). Expression of both enzymes takes place in the form of a single chain precursor. It contains the sequences corresponding to subunits, the prosegment sequence (in the N-end of the larger subunit) and the PSI (plant-specific insertion) sequence. The PSI sequence probably plays a role during precursor targeting, e.g. to the vacuole or apoplast (Ramalho-Santos et al. 1998; Faro et al. 1999).

Another type of protease has been isolated from non-pollinated ovaries of pea (Pisum sativum). Thanks to the application of successive column chromatography techniques, a 77-fold purification of the enzyme was obtained and its molecular mass was established at ~30 kDa on the basis of electrophoresis under denaturing conditions (SDS-PAGE). Protease activity was significantly inhibited by leupeptine, E-64 [L-(trans)-epoxysuccinyl-leucylamido (4-guanidino) butane] and iodoacetamide, which indicates its belonging to the cysteine catalytic class. The enzyme exhibited optimum action in a very wide pH range of ~7.0. By using immunocytochemical methods it was found to accumulate in the vacuoles and the tonoplast of exocarp cells and the outer layer of mesocarp cells of the ovary, especially those senescing, which seems to indicate the functions played by this enzyme (Cercos and Carbonell 1993, Cercos et al. 1993).

Chen and Foolad (1997) cloned and characterized a gene encoding an aspartic protease-like protein detected in the ovary of barley (Hordeum vulgare). Its putative amino acid sequence has motifs with a large homology to aspartyl proteases, especially close to active sites, although the whole sequence is not very similar to known enzymes from that class. The putative enzyme is distinguished by the fact that as the only plant aspartyl protease it does not have a prosequence or PSI. This suggests that protease is subjected to a different post-translation control and probably is not targeted to the vacuole. Nucellin, as this protease used to be called, has weak expression in nucellus cells several days before and 10 days after pollination, whereas 3–4 days after pollination respective mRNA transcripts are numerous. Synchronization between high nucellin expression and the degradation of nucellus cells indicates its involvement in PCD and/or functions played in the nutrition of fertilized ovules.

While searching for genes with increased expression in the pistils and ovaries of rice (Oryza sativa) immediately before and during flowering, it was found that one cDNA fragment encodes an amino acid sequence very similar to the sequences of known plant subtilisin-like serine

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proteases (RSP1). Proteases from that subgroup are synthesized as inactive preproenzymes and are targeted outside the cell. Their processing consists of the cleavage of 28 amino acid units from the peptide N-end during transport, and next the 99-amino acid prosequence. The transcripts corresponding to RSP1 were also detected in filaments of stamens by *in situ* localization analysis. This suggests the involvement of RSP1 – along with PR (pathogenesis-related) proteins – in defense processes, including also that against the incompatible pollen. An immediate degeneration of the stigma, pistil, ovary and anther cells occurs after successful pollination and RSP1 seems to be involved in PCD. Extracellular subtilisin-like proteases are considered to be responsible for processing of signal peptides. In order to decide whether RSP1 is involved in such processes, it is necessary to identify its potential substrates (Yoshida and Kuboyama 2001).

**Pollen proteases**

The presence of proteolytic enzymes in pollen was detected as early as over 100 years ago (Green 1894). Since then it has been repeatedly confirmed with cytochemical and electrophoretic methods (Kammann 1904; Paton 1921; Bellartz 1956; Knox and Heslop-Harrison 1969; Bhalla et al. 1986). Proteases have been localized most frequently in the pollen wall, from which they are easily washed (Knox et al. 1975), in pollen secretions during hydration (Baraniuk et al. 1988) and in the germinating pollen tube (Stanley and Linskens 1985). Considerations of the role of proteolytic enzymes during processes of pollen-pistil interaction were initially based on the application of exogenous proteases in experiments (Hiscock and Dickinson 1993; Stead et al. 1980, Roberts et al. 1980), as the knowledge on native pollen enzymes was very limited at that time. However, many troublesome pollen allergens were found to exhibit hydrolytic properties, including proteolytic ones (Bousquet et al. 1978), which has resulted in an increased interest in this group of enzymes in recent years. Basic information on purified pollen proteases is presented in Table 1.

By the use of column chromatography, including high-pressure liquid chromatography (HPLC), an enzymatic preparation was obtained from pollen of maize (*Zea mays*) line Co255, with the specific activity 52 times higher than that in crude extract. Electrophoreses under non-denaturing and denaturing conditions (PAGE and SDS-PAGE) made it possible to state that it is a monomer with a molecular mass of ~21 kDa. Investigations on the effect of specific inhibitors on the activity of the purified enzyme showed that only leupeptine and especially phenylmethylsulfone fluoride (PMSF) very significantly inhibited hydrolytic properties of the protease. This suggested the presence of serine in the active center. Optimum activity of the protease is found in the temperature range of 45–50°C and at pH 4.8. The latter value is surprisingly low and differs from the pH values generally assumed as optimal for the action of serine enzymes (usually weakly basic). However, it needs to be remembered that some plant serine proteases exhibit optimum activity at acidic pH. Neither the activity of this enzyme nor its thermal stability have been influenced by divalent metal ions, although this is commonly observed for serine proteases in presence of calcium ions.

The investigated enzyme cannot be removed from pollen grains by washing, indicating its location inside pollen structures (Radowski et al. 1994).
effect of the presence of pepstatin and dipicolinic acid on proteolytic activity showed a strong negative effect of the presence of pepstatin and dipicolinic acid. Studies on the commonly used inhibitors showed that the protease was composed of two identical subunits. The applied HPLC column showed that it had a mass of ~60 kDa, which suggested that it was a single band. However, a short digestion of the peptide with a non-specific pronase R from the bacterium Streptomyces griseus resulted in the formation of a product with a lower molecular mass. Aspartyl protease isolated from maize pollen towards a purified inhibitor from the fungus formed a peptide with a molecular mass of ~3 kDa. The aspartyl enzyme exhibited the optimum catalytic properties, and its catalytic classes were similar to those used in the case of the previous enzyme. However, the enzyme retained on the column varied depending on species, and high in grasses, including maize. Maize pollen grain, with the average diameter of 90 μm, germinated into the pollen tube of several dozen cm in length in a very short time. Barnabas and Fridvalszky (1984) reported the growth rate close to 1 cm per hour. Thus it seems natural that the intensification of metabolic processes occurring there is rapid and requires prompt mobilization of proteins for synthesis.

Another proteolytic enzyme was also isolated from microgametophytes of maize line Co255. After purification, the optimal conditions of its catalytic properties were found. The applied chromatographic methods were very similar to those used in the case of the previous enzyme and resulted in a similar purification ratio (46 times). As a result of electrophoretic separation of the other protease from maize pollen under denaturing conditions, a single band was obtained, corresponding to a molecular mass of 33 kDa. However, the enzyme retention time from the applied HPLC column showed that it had a mass of ~60 kDa, which suggested that the protease was composed of two identical subunits. Studies on the commonly used inhibitors of proteolytic activity showed a strong negative effect of the presence of pepstatin and diazoacetylorn-leucine methyl ester, indicating that this enzyme belonged to the aspartyl catalytic class. The aspartyl enzyme exhibited the optimum action at pH 5.6 and temperature of 45°C.

Surprising results were brought by studies on the effect of protease activity at the presence of bacitracin. They were found at pH 5.9 for serine protease and at pH 5.0 for the aspartyl enzyme. Two-dimensional electrophoresis (2-D) showed that aspartyl protease selectively hydrolyzed proteins from the stigma exudate. It is difficult to determine unambiguously whether the enzyme supplies in this way substrates necessary during the germination and growth of the pollen tube, facilitates the penetration of the pollen tube through the transmission tissue of the pistil, or maybe plays other functions. However, the involvement of aspartyl protease in the process of pollen grain germination is obvious (Radowski et al. 1996).

Both proteases isolated from maize pollen differed in some of their properties from typical enzymes classified to the serine and aspartyl catalytic classes. Another confirmation of this observation was the effect on the activity of pollen enzymes caused by bacitracin, a peptide antibiotic considered to be a universal protease inhibitor. In contrast to enzymes from animal tissues, fungi and bacteria, as well as those extracted from diploid tissues of plants, both proteases from maize pollen were significantly stimulated by bacitracin even in the presence of their other inhibitors (PMSF and pepstatin, respectively).

A similar effect of bacitracin was also observed on proteolytic enzymes from pollen of other plants belonging to the family Poaceae, such as orchard grass (Dactylis glomerata) or Festulolium (a hybrid of Lolium multiflorum and Festuca pratensis). Tested proteases from tobacco pollen (Nicotiana tabacum and N. alata) increased their activity only slightly or did not change it at all. Proteolytic enzymes from dwarf mountain pine pollen were inhibited in the presence of bacitracin. The stimulating effect on the activity of both proteases from maize pollen was unexpected and
its mechanism is at present difficult to interpret. It may be the effect of slight allosteric changes close to the substrate-binding site, which may accelerate the hydrolysis of the peptide bond (Radłowski et al. 2005). It is noteworthy that the expression of some plant genes occurs only at the microgametophyte phase, and the observed differences strongly suggest that this group may include genes of pollen proteases (Mascarenhas 1990; Ottaviano and Mulcahy 1989).

Two proteolytic enzymes were also isolated and purified from microgametophytes of mesquite (Prosopis velutina) by Matheson et al. (1995). Both proteases belong to the serine catalytic class and peptidase IImes in addition to usual endopeptidase properties has also the properties of aminopeptidase. Electrophoresis under denaturing conditions of peptidase IImes revealed the presence of a single band, corresponding to a molecular mass of 84 kDa. The enzyme is thermally very stable, its optimum action is found in a wide range of pH values of 7.5–9.0, and the presence of calcium ions has a stabilizing effect. The isolated enzyme very effectively digests short peptides on the carboxyl side of basic amino acids, with huge preference of arginine (slightly lower of lysine), while it degrades protein with a low efficiency. Enzymatic activity of peptidase IImes is not inhibited by many typical trypsin inhibitors, and its N-end is blocked. Several differing properties exhibited by this enzyme convinced those authors to assume that the mechanism of its action does not completely correspond to that of typical serine class proteases.

There is no information concerning physiological functions played by peptidase IImes in pollen, although its high specificity suggests that they may be very subtle, e.g. in processing of peptides during adhesion of pollen with the stigma. Peptidase IImes, similarly to the one discussed previously, poorly hydrolyses protein, while small peptides are digested by it with a high efficiency. Its molecular mass is 92 kDa, optimum pH values fall within the range of 7.5–9.5 and when stored at –20°C it does not change its activity for many months. The presence of calcium ions does not have any effect on its stability and activity. Due to the blocked N-end it was possible to determine the amino acid sequence of only a small internal fragment. Similarly to peptidase IImes, peptidase IImes exhibits a very high specificity of action, preferring the side of the alanine carboxyl group (when it acts as endopeptidase) and phenylalanine when it performs the functions of aminopeptidase (Matheson and Travis 1998).

From pollen of ragweed (Ambrosia artemisiifolia) the next two proteolytic enzymes were purified, belonging to the serine catalytic class according to the inhibitory studies (Bagarozzi et al. 1996). One of them, chymotrypsin-like, similarly to the previously described proteases from mesquite pollen, degraded only short peptides, and among the tested proteins only serpin α-1-PI was hydrolyzed and its chain was cleaved only at one site. Both gel filtration and SDS-PAGE showed that the enzyme has a molecular mass of 82 kDa, and its optimum activity is reached at a definitely basic pH value of 9.0. The protease is thermally stable – stored for many months at the temperature of –80°C it loses less than 2% of its original activity. The established 17-amino acid N-end sequence of the polypeptide chain is not homologous with the sequence of any other known protease. The enzyme exhibited a very high specificity of action, preferring in the P1 site mainly phenylalanine and, to a lesser extent, leucine and methionine. Hydrolyzed peptides need to be of an appropriate length (at least four amino acids). This is probably connected with precise requirements in the protease substrate site. It seems likely that the enzyme is involved in the germination of pollen tube and/or the pollen-pistil interaction, through specific digestion of precursor proteins and processing of signal peptides.

The other protease isolated from the same source, in spite of numerous similarities, has several properties enabling its distinguishing from the above-mentioned enzyme (Bagarozzi et al. 1998). It belongs to a subgroup of trypsin-like serine class and has a blocked N-end of the chain. Its molecular mass and optimum pH are similar to the values obtained for the previous enzyme, amounting to 80 kDa and pH of ~9.0, respectively. The presence of calcium ions stabilizes the enzyme, which does not seem capable to hydrolyze large proteins but only short peptides (e.g. dipeptides). It must be mentioned that natural protein (peptide) substrates of most pollen proteolytic enzymes are still unknown. Many of the typical inhibitors of serine proteases (e.g. Kunitz-type trypsin inhibitor, some serpins) have no effect on its activity, while others (coumarin derivatives) are very effective. Protease preferentially degrades substrates having arginine in the P1 position. Both proteases found in ragweed pollen in approximately identical concentrations do not interact during degradation.
A very interesting proteolytic enzyme is the one localized in pollen of timothy grass (Phleum pratense). Protease is a glycoprotein, whose molecular mass was determined at ~35 kDa (Grobe et al. 1999). The activity of the freshly isolated enzyme (nPhl p 1) is weak and requires a preincubation in an activation buffer (containing cysteine) under reducing conditions. Self-digestion of the N-end prosequence results in the obtaining of full activity by nPhl p 1. Investigations on the effect of the presence of numerous protease activity inhibitors showed that the activity of Phl p 1 is primarily affected by inhibitors of the cysteine class (but not E 64) and – to a lesser degree – of the serine class (e.g. PMSF). The inhibitory effect was also found for high concentrations of copper ions. A similar case of inhibition caused by effectors specific for two different groups may be observed also for papain and protease from the dust mite (Dermatophagoides pteronyssinus). A comparison of the amino acid sequences close to the functional sites of papain and nPhl p 1 made it possible to assign finally the enzyme from timothy grass pollen to the cysteine catalytic class. The protease exhibited optimum action at acidic pH of 3.6–6.0 and preferred hydrolysis of the peptide chain on the carboxyl side of arginine. The presence of a prosequence in its structure suggests that it belongs to enzymes located in the apoplast. Its amino acid sequence showed over 90% homology to β-expansins from pollen of other grasses. In spite of numerous similarities of nPhl p 1 to cysteine proteases of group C1, it exhibits such significant differences that it may be assumed to belong to a new subgroup of enzymes, which had a common origin with C1 enzymes. This is indicated by a still low homology of the whole amino acid sequence, a lack of the ERFNIN motif in the prosequence, which is distinctly shorter, reminding cathepsin B in this respect. Protease from pollen of timothy grass, similarly as other β-expansins, degrades extensin proteins, loosening the structure of the cell wall, thus facilitating its growth.

Proteolytic enzymes play also a very significant role during microsporogenesis. In Easter lily (Lilium longiflorum), one of the proteins specific for this stage of pollen formation, LIM9, is synthesized in the form of a preproprotein. Going through the endoplasmic reticulum (ER) and the Golgi body, it is glycosylated and in this form released to the anther loculus. The expression of processed protein with a molecular mass of 82 kDa takes place in the tapetum cells, and its accumulation is observed especially when microsporocytes reach the tetrad stage. Analyses of zymograms and a similarity of the amino acid sequence in the active site region show that LIM9 belongs to the class of subtilisin-like serine proteases. However, the sequence of the whole molecule indicates a slight homology to typical serine enzymes. Tissue expression of LIM9 is closely correlated with its functions. One of them is its involvement in the formation of gaps in the primary cell wall of microsporocytes and the loosening of the extracellular matrix, facilitating the access of β-(1-3)-glucanase to the callose layer. Another role played by LIM9 may be the destruction of tapetum cells, which leads to the discharge of mature pollen grains. Equally significant seems to be the postulated function at one of the stages in signaling through the formation of natural, bioactive peptides or peptides produced after a pathogen attack. It is worth mentioning that according to analysis of zymograms a large amount of another proteolytic enzyme with a molecular mass of 60 kDa is present in the tetrad stage (Taylor et al. 1997). An accumulation of mRNA of protein TA56 was observed during microsporogenesis in tobacco (N. tabacum) and the amino acid sequence of this protein exhibits a very large similarity to proteases of the cysteine class (Koltunow et al. 1990).

Jamoom et al. (2001) suggested that the cytoplasmic male sterility in maize, manifested in the formation of collapsed pollen, may be the effect of the action of protease with a molecular mass of 50 kDa, not found in normal pollen.

Pollen may cause many diseases of the respiratory tract. This has resulted in understandable interest in its allergens, among which many exhibit enzymatic activity, including proteolytic one. Functional properties of many known pollen allergens have been collected and described by Bufe et al. (1996) and Raftery et al. (2003).

**Proteolytic enzymes in pollen-pistil interaction**

The activity of enzymes and their interaction with their corresponding substrates affect all phenomena taking place during the pollen-pistil interaction (de Graaf et al. 2001). Pollen proteolytic enzymes, as well as those located in the female generative organs, actively participate in the processes of adhesion, hydration, signaling,
germination of the pollen tube and its growth into the transmission tissue of the pistil. The proteolytic digestion of pellicle proteins of the stigma reversibly decreases pollen adhesion, especially at its later stages (Swanson et al. 2004; Stead et al. 1980; Luu et al. 1997). This suggested a formation of complexes composed of the pollen coat/wall proteins and pellicle polypeptides, which facilitate hydration of compatible pollen. A lack of one component as a result of the protease action would cause a rejection of pollen as incompatible (Roberts et al. 1980). Another possibility would be the damage of proteins and enzymes present on the surface of the stigma, activating pollen cutinase necessary for the degradation of the cuticle and the growth of the pollen tube into the transmission tissue (Hiscock and Dickinson 1993). The experiments described above were conducted with the use of exogenous proteolytic enzymes. However, experiments with native aspartyl protease, located in the pollen wall, made it possible to assume that it has similar functions (Radłowski et al. 1996).

Cardosin A, as the only protease of the aspartyl class, contains the RGD motif, characteristic of proteins recognized by surface receptors from the integrin group. The RGD sequence is located in cardosin A on the side opposite to the active site, which facilitates adhesion to pollen protein with a molecular mass of 100 kDa, its probable receptor. This suggests the involvement of this enzyme in the RGD-dependent proteolytic mechanism during pollen recognition and its germination (Frazao et al. 1999; Faro et al. 1999; Simoes and Faro 2004). The location of cardosin B and other proteolytic enzymes in the transmission tissue of the pistil may indicate their role in the reorganization of the apoplasm to promote the growth and nutrition of the pollen tube (de Graaf et al. 2001; Vieira et al. 2001). It is interesting that a similar action of aspartyl protease on stigmatic silk proteins as that described by Radłowski et al. (1996) was postulated by Zinkl et al. (1999).

The presence of ubiquitin and its related proteins was found in the pollen of numerous plants, although there are contradictions concerning the level of its expression (Callis and Bedinger 1994; Kulikauskas et al. 1995; Alche et al. 2000). The ubiquitin/26S proteasome protein degradation pathway may play a significant role during the pollen-pistil interaction in the self-incompatibility mechanisms. Self-incompatibility (SI) is a process preventing inbreeding, making the pistil capable of rejecting the pollen coming from the same individual. Pistil S-RNases are responsible for gametophytic self-incompatibility in some plants (family Solanaceae, snapdragon, Japanese apricot, almond). The pollen determinants of gametophytic SI are proteins containing the F sequence (F-box) belonging to one of five classes of E3 proteins (ubiquitin-protein ligases) closely involved in the process of ubiquitination of proteins to be degraded. The SCF complex does not mark their S-RNases, they are not subjected to proteolysis and degrade RNA from the pollen tubes of “their” pollen. In the case of foreign pollen, S-RNAs are degraded, they do not damage pollen RNA, and the pollen tube itself may grow in the direction of the ovary (Hare et al. 2003). In other plants, e.g. from the family Brassicaceae, sporophytic SI is observed, occurring on the surface of the stigma. In this case the interacting elements are pollen peptides called SCR/SP11 and protein kinases located in the stigma: SRK (S-Locus Receptor Kinase) and the ARC1 (Arm Repeat-Containing) proteins cooperating with kinases. The latter contain a U-box sequence and have the activity of ligases E3. When pollen is recognized as incompatible, ARC1 proteins are immediately phosphorylated. This promotes protein degradation and switches on the signaling pathway, which causes the closing of aquaporins on the surface of the stigma, preventing pollen hydration and its germination (Hare et al. 2003; Shiba et al. 2001). The involvement of ARC1 proteins in the incompatibility mechanism is evidenced by the fact that no increase in the abundance of ubiquitinated proteins is observed as a consequence of incompatible pollination in the pistils of transgenic plants containing antisense mRNA of this protein and by a disturbance of SI in the presence of specific proteasome inhibitors (Stone et al. 2003). While characterizing pollen proteases it was already mentioned that some of them are not capable of degrading large proteins or exhibit a high specificity of the peptide chain hydrolysis site (Radłowski et al. 1994b, 1996; Matheson et al. 1995, 1998; Bagarozzi et al. 1996, 1998; Widmer et al. 2000). Their involvement in the processing of SCR/SP11 peptides seems very likely.

Programmed cell death (PCD) consists in selective elimination of unwanted cells in the organism and is a necessary element of the developmental processes. Three stages may be distinguished in PCD: induction of a signal, its transmission and execution. Proteolytic enzymes
are involved in each of the stages (Beers et al. 2000). Functions of organs connected with reproduction probably depend mainly on PCD. The generation of functional pollen simply requires the death of tapetum cells, and sporogenesis in female organs very often begins together with the death of all, except one, cells produced as a result of meiosis. The female tissue interacting with compatible pollen quickly degenerates to make it easier for the pollen tube to reach the ovule (Yoshida and Kuboyama 2001). In the case of incompatible pollination both tissues interact in the elimination ofthe inappropriate pollen. Another aim of the disintegration of cells in generative organs is to supply substrates for the developing pollen grains, the embryo sac or the growing pollen tube (Wu and Cheung 2000). Most frequently, the involvement of cysteine caspases (in plants rather caspase-like) and aspartyl class enzymes is suggested in PCD, although an increased mRNA level or increased activity of proteases belonging to the other catalytic classes were also observed in degenerating generative tissues (Beers et al. 2000).

Concluding remarks

It seems that proteolytic enzymes from generative parts of plants perform essential functions during pollination. Starting from the immobilization of pollen grains, through hydration, signaling, germination and growth of the pollen tube, and finishing with the fertilization of the ovule, the action of specific proteases affects proper functioning of these processes. For many years proteolytic enzymes from generative tissues, especially pollen, were not considered to be of interest. However, pollen causes numerous allergic disorders, such as hay fever or asthma, which in some countries affect up to 25% of the adult population. The health protection and economic reasons considerations have resulted in an increased interest in this group of microgametophyte hydrolases, because many of pollen allergens exhibit proteolytic activity. We may only regret that the allergenic properties of pollen proteases were most frequently focused on, with no attention paid to their physiological functions. The discussed enzymes in a vast majority of cases differ from typical proteases included in the same catalytic classes. This pertains to the effect of some characteristic inhibitors and metal ions on their activity, optimum pH, specificity of their action towards specific substrates, a lack of certain amino acid sequences or the presence of exceptional sequences, etc. This has resulted in hypotheses that at least in several cases they may have a slightly different catalytic mechanism than it has been universally assumed. The above-mentioned differences show also that genes coding for proteases, especially the pollen ones, are expressed only in a specific tissue or at a certain developmental phase. However, the presence of some proteolytic enzymes in generative organs of plants was inferred from the similarity in the cDNA sequence. On this basis the sequence of the active protein was deduced. This approach does not make it possible to investigate interesting physico-chemical properties of the enzymes, and the determination of their functions is highly speculative. A likely aim of studies in the near future will thus be to gain insight into their native substrates, which will make it possible to understand better the role of proteases in processes taking place during pollination.

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