

Rapid report

Differential influence of bacitracin on plant proteolytic enzyme activities

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Abstract

The effect of bacitracin on the activity of proteases extracted from pollen and sprouts of various plant species and compared to five commercially available proteases was studied. Bacitracin stimulates some pollen proteolytic enzyme activities, contrary to its inhibitory influence on proteases from the other sources. Proteases from maize pollen, inhibited by pepstatin and phenylmethylsulfonyl fluoride, immediately accelerate their activities after addition of bacitracin to the reaction mixture. The stimulating influence of peptide antibiotic on pollen proteases of some plants is unexpected and molecular mechanism of this phenomenon requires a further elucidation. The augmentation of allergenic response caused by pollen enzymes and drugs containing bacitracin is discussed.

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Bacitracin, an antibiotic produced by bacterial strains *Bacillus subtilis* and *B. licheniformis*, is a 12-amino-acid peptide that contains a heptapeptide cyclic structure and a thiazoline ring. Crude bacitracin is a mixture of over 30 congeners, which differ in their amino acid compositions [1]. Antimicrobial activity and mechanism of activity of antibiotic bacitracin particularly against gram positive bacteria, in which peptidoglycan is a major component of cell wall, have been reported [2]. Bacitracin influences also the accumulation of cell wall intermediates, amino acid incorporation and cell lysis [1]. The biological activity of bacitracin is associated with binding of divalent metal ions to this particle [1]. The antibiotic is known as well as a strong inhibitor of all classes of proteases [3] and it is resistant to enzyme digestion [4]. This resistance is probably caused by the presence of thiazoline ring within the antibiotic particle. The inhibitory effect of bacitracin on protease activities is not related with its metal binding capability. Hay fever and asthma are two of the most

widespread and important allergic diseases. Both can be easily triggered by inhalation of pollutants such as, e.g., dust mites, fungal and bacterial secretion and plant pollen [5]. Several allergens possess proteolytic activity [5–8]. This caused that isolation and studies of pollen proteases became a focus of interest and yielded a purification of the enzymes from pollen grains of mesquite, ragweed and grasses [6–8].

The plant proteolytic enzymes were found in practically all tissues [9]. Serine and aspartic classes of protease activities were observed in maize pollen [10]. The aim of this study was to investigate the effect of bacitracin on proteases from pollen and plant vegetative tissues, which was compared with the commercially available proteases purified from animal, fungi and bacteria. The enhancement of pollen protease activities in the presence of bacitracin could accelerate the allergenic response.

Pollen grains from maize (*Zea mays* L.), *Festulolium* (intergeneric hybrid of *Lolium multiflorum* L. × *Festuca pratensis* Huds.) and cocksfoot (*Dactylis glomerata* L.) as well as tobacco (*Nicotiana tabacum* L. and *Nicotiana glauca* L.) were collected from plants growing in a greenhouse as described Stanley and Linskens [11]. Pollen from dwarf mountain pine (*Pinus mughus* Scop.) was derived from one individual growing in Tatra region in

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season 2002. Pollen samples were stored in tightly closed vessels at -20°C . The seeds of lupine (*Lupinus luteus* L.), potato (*Solanum tuberosum* L.) and maize were sprouted in moist, sterile quartz sand at 22°C in the dark. The shoots were cut off after 3 days and used for proteins extraction. Three-day-old sprouts of lupine, potato and maize were suspended (1:5, w/v) in 100 mM Tris–HCl buffer pH 7.5, 1 mM MgCl_2 , 0.1 mM 2-mercaptoethanol and 10% glycerol (v/v) (buffer A) and ground with a pestle three times for 10 min each with 5-min intervals. The resulting slurry was centrifuged at $14\,000\times g$ and supernatant loaded on Sephadex G-25 column ($1\times 12\text{ cm}$), equilibrated previously with 2–3 bed volumes of the same buffer. Proteins were eluted from the column in the void volume. All procedures were made at 4°C . The fractions containing proteolytic activities were used for enzyme proteolytic activity experiments. Total protease activities from plant pollen were obtained in the similar way. Extraction of serine protease and elution of aspartic protease from maize pollen wall were carried out as described previously [10]. The proteolytic activity was mainly determined by the fluorometric method with fluorescein isothiocyanate-labelled casein (FITC-casein) as a substrate [12]. Occasionally, the influence of bacitracin (Sigma Chemical Co., St. Louis, MO, USA) on protease activities was confirmed in the reaction with azocoll and azoalbumin (Sigma) [13]. In all cases the reaction mixture was pre-incubated at 22°C for 1 h before the hydrolytic reaction in the presence of bacitracin. The experiments were repeated three times in duplicate and results presented are averages of received data. In experiments described here, we tested the effect of bacitracin on commercial trypsin and chymotrypsin from bovine pancreas (Sigma), pepsin from hog stomach (Fluka Chemie AG, Buchs, Switzerland), proteinase K from *Tritirachium album* (Fluka Chemie) and pronase CB and R from *Streptomyces griseus* (Calbiochem, La Jolla, CA, USA). The enzymes and bacitracin were dissolved in 0.1 M Tris–HCl buffer pH 7.5 except pepsin dissolved in 0.1 M acetate buffer pH 5.2. Concentration of the proteases as well as reaction time were chosen as given by Twining [12]. In controls, the enzymatic solution was replaced by the same volume of the buffer. The other blank assays contain bacitracin but no enzyme. All controls were treated as the assays. The results presented are the differences of subtraction of blanks from each experimental value. One unit (U) of protease activity was defined as the amount of enzyme digesting $1\text{ }\mu\text{g}$ FITC casein h^{-1} [14]. Protein concentration was determined according to Bradford's method [15]. Data were expressed as the means $\pm$ standard deviation (S.D.) of three independent experiments made in duplicate.

The activity of all commercial proteases as well as of those extracted from plant sprouts and from dwarf mountain pine pollen was inhibited by bacitracin, as shown in Table 1. However, a highly stimulatory effect

Table 1

Effect of bacitracin on protease activities

Protease or its source	Activity (%)
Pepsin	79.6 ± 3.2
Trypsin	23.1 ± 4.1
Chymotrypsin	45.4 ± 7.2
Proteinase K	75.5 ± 6.1
Pronase CB	44.7 ± 5.9
Pronase R	77.1 ± 2.8
Lupin sprouts	35.2 ± 3.4
Potato sprouts	55.8 ± 4.8
Maize sprouts	65.3 ± 4.6
Pollen from maize inbred line S339	187.4 ± 5.5
Pollen from maize inbred line Co255	180.7 ± 6.3
Eluted from pollen	199.6 ± 6.1
Non-eluted from pollen	162.8 ± 7.3
Pollen from Festulolium	173.0 ± 8.0
Pollen from cocksfoot	144.8 ± 5.6
Pollen from tobacco (<i>Nicotiana tabacum</i>)	107.1 ± 4.6
Pollen from tobacco (<i>N. alata</i>)	103.3 ± 2.8
Pollen from dwarf mountain pine	75.7 ± 3.4

Activity of the enzyme without bacitracin in the assay was assumed to be 100%. In experiments the final concentration of bacitracin was 5 mM. The protein fraction containing proteolytic activity was eluted from pollen grains of maize Co255 inbred line as described previously [10]. The presented data are means of three experiments made in duplicate $\pm$ standard deviation.

of the antibiotic on the activity of proteases from pollen of plants from the Poaceae family (maize, Festulolium and cocksfoot) was observed, whereas in the case of tobacco pollen enzymes no or very slight influence was found. The highest concentration of bacitracin used in the experiment (final concentration 20 mM) did not inhibit the activity of the maize pollen enzyme but approximately 90% of the commercially available chymotrypsin activity was inhibited by bacitracin (Fig. 1). The optimal stimulatory effect of antibiotic on the maize pollen enzyme may be noticed in a concentration range from 1 to 10 mM. The velocity of FITC-casein digestion by aspartic protease eluted from the maize pollen is inhibited in the presence of pepstatin (final concentration $5\text{ }\mu\text{g/mL}$, Sigma). The addition of bacitracin (final concentration 1 mM) increases the velocity of hydrolysis even with pepstatin in the reaction mixture (Fig. 2). In the experiment with non-eluted serine protease and phenylmethylsulfonyl fluoride (PMSF; final concentration, 1 mM; Sigma), a similar influence of bacitracin has been observed (Fig. 3). However, the effect of antibiotic on enzyme activity was lower than in the previous case and the maximal velocity of FITC-casein proteolysis reached only the level obtained without PMSF in the assay. Bacitracin has antimicrobial activity and as well acts as an inhibitor for proteases of all classes [3]. However, the model of enzyme inhibition is recognized in detail only for one serine protease, savinase [16]. In our experiments we found the influence of bacitracin on pollen proteases as an enhancer, but not as an inhibitor. Detection of the stimulatory effect of bacitracin on activities of serine as well as aspartic

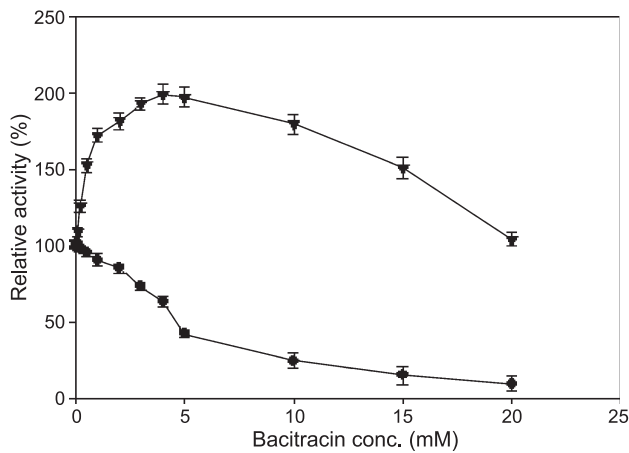


Fig. 1. Influence of different concentrations of bacitracin on the activity of chymotrypsin (●—●) and aspartic enzyme from maize pollen-coat (▼—▼). Each assay contains 1 μ g of chymotrypsin or 4.6 μ g of protein eluted from maize pollen grains of Co255 inbred line, respectively. Data are means $\pm$ S.D. ($n=6$). Activity of the enzyme without bacitracin in the assays was assumed as 100%.

proteases from maize pollen is exceptional and fascinating. Most probably this phenomenon could arise from some minor rearrangement close to the substrate-binding site of the enzyme. The interaction of bacitracin with pollen protease could change an allosteric conformation and, in this way, could facilitate the hydrolysis. The presence of the antibiotic in the reaction mixture causes unusual effects on the activities of pollen proteases from other plants of Poaceae family (stimulation) and tobacco (none or very slight effect). Proteolytic activities from plant vegetative tissues and extracted from dwarf mountain pine pollen are inhibited by bacitracin, so they seem to act as typical proteases from bacteria, fungi and animals (Table 1). In our work, bacitracin was studied as a strong inhibitor of

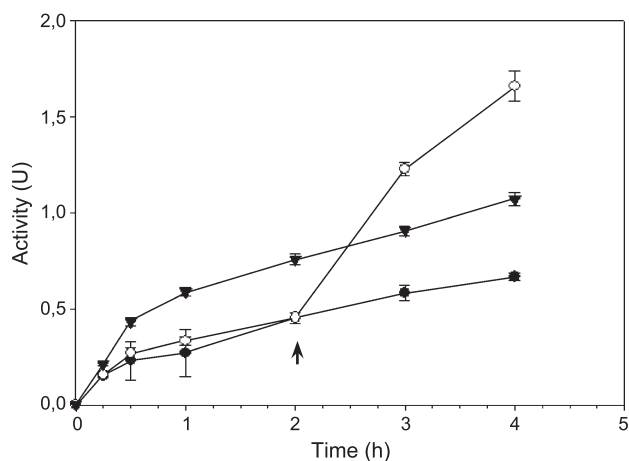


Fig. 2. Time course of FITC-casein digestion by aspartic protease from maize pollen; assays without pepstatin and bacitracin (▼—▼); pepstatin in the assays (●—●); bacitracin added (arrow) after 2 h to the assays with pepstatin (○—○). Each assay contains 4.6 μ g of protein eluted from maize pollen grains of Co255 inbred line. Data are means $\pm$ S.D. ($n=6$).

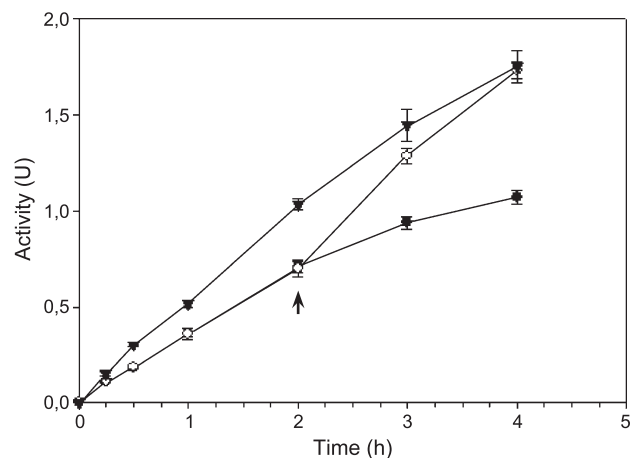


Fig. 3. Time course of FITC-casein digestion by serine protease from maize pollen; assays without of PMSF and bacitracin (▼—▼); PMSF in the assays (●—●); bacitracin added (arrow) after 2 h to the assays with PMSF (○—○). Each assay contains 3.5 μ g of protein non-eluted from maize pollen grains of Co255 inbred line. Data are means $\pm$ S.D. ($n=6$).

proteases, not as an antibiotic; for this reason, its concentrations used in experiments seem rather high in comparison to the minimal inhibitory concentration (MIC). The highest final concentration of bacitracin used in the experiments (20 mM) caused almost complete inhibition of chymotrypsin activity, whereas the action of aspartic protease eluted from maize pollen was still slightly stimulated (Fig. 1). This is a commonly used practice in experiments made in vitro, due to the amount of the enzymes many times larger in the assays than in the original tissue. Similar concentrations of bacitracin were used in the reaction mixture by van Noort et al. [17] in the investigations with serine and aspartic protease from *Aspergillus niger*. Furthermore, some other protease inhibitors, e.g., ethylenediaminetetraacetic acid (EDTA), PMSF, iodoacetamide and 2,2 dipyridyl disulfide, are commonly used in the final concentration of 1–5 mM [18]. The stimulating influence of the antibiotic studied on the aspartic protease activity washed from pollen-coat of Co255 maize inbred line was observed, even in the presence of pepstatin, another inhibitor of aspartic enzymes (Fig. 2). The kinetics of FITC-casein hydrolysis determined in the presence of pepstatin, after the addition of bacitracin, immediately rises above the control kinetics carried out without pepstatin in the reaction mixture. It might suggest that the influence of bacitracin on the enzyme activity is much stronger than that of pepstatin, even in its optimal concentration. The activity of serine protease from the maize pollen grains is strongly inhibited in the presence of 1 mM PMSF. After the addition of 1 mM bacitracin to the assay, the velocity of FITC-casein proteolysis reaches the same level as in the case of control without PMSF (Fig. 3). It is known [16] that the antibiotic binds to savinase (serine protease) in an equimolar ratio (2:2) and interacts with the catalytic site of one molecule and the substrate-binding site of another. The stimulatory

effect of bacitracin on the activities of both maize pollen enzymes is unexpected and its mechanism at the moment is difficult to explain. We did not find any information concerning the mechanism of the studied peptide action on the cysteine and aspartic proteinase activity. Bacitracin inhibits also the metalloproteinase [18], however, it is not related to its metal-binding properties. The pollen proteases are different in comparison with those from other sources such as vegetative plant tissues, animals, fungi and bacteria [7,10,14,20]. It must be emphasized that some plant genes are expressed only in the microgametophyte stage [21,22]. The distinct influence of bacitracin on the pollen protease activities suggests that these enzymes are the products of such genes. We have previously described the presence of serine and aspartic proteases in pollen grains of maize [10]. Both enzymes differ in regard to the structure, conditions of optimal activity and location in the pollen grain. Aspartic protease, easy to wash from the pollen grains, takes part in the first stage of pollen tube germination. Zinkl et al. [23] also suggested that the pollen aspartic enzyme acts during the pollen–stigma interaction. Adhesion between the pollen and stigma cells is highly selective. The pollen proteases, similarly as in animal embryo, reorganize the extracellular matrix [24]. Low molecular mass peptides and peptide-containing compounds, present also in pollen and/or stigma, might change the protease activities and influence the pollination [25]. It is possible that bacitracin could be a good tool for studies of such effect [26–28]. It is also worth emphasizing that pollens are important triggers for allergic diseases. Many of the pollen allergens are enzymes. The activity of proteolytic enzymes from exogenous sources may disorder a subtle equilibrium of host proteinase/proteinase inhibitor and may cause a chronic inflammation of the upper airways of the lungs [5]. Grobe et al. [29] suggest that the exogenous proteolytic activity may lead to enhanced mobility of the allergen because “the enzymatically active allergen may elicit allergic responses through interaction with regulatory molecules of the immune system”. The increase of pollen proteases activities in the presence of bacitracin could augment or accelerate the allergenic response [19] and therefore may have a potential physiological significance in the case when bacitracin in treatment is used.

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