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Isolation and partial characterization of an acid phosphatase from *Artemisia vulgaris* pollen extract

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Abstract: An acid phosphatase from an extract of mugwort (*Artemisia vulgaris)* pollen was purified by a factor of 48 by a combination of ion exchange and gel-chromatography. The molecular weights of the enzyme were 76 kDa and 73 kDa, determined by gel filtration on a Sephadex G-100 sf column and by SDS PAGE (under reducing and non-reducing conditions), respectively. In analytical isoelectrofocusing, the enzyme appears as two very close bands, pI at about 4.2. The optimum pH for the enzyme is 5.4. The apparent K_m for *p*-nitrophenyl phosphate was estimated to be 0.16 mM. The purified enzyme has broad specificity, and hydrolyses *p*-nitrophenyl phosphate and α-naphthyl phosphate. Pyrophosphate and *O*-phospho-L-tyrosine were estimated to be the best substrates for this enzyme as potential *in vivo* substrates. The enzyme is inhibited competitively by phosphate (K_i = 1.25 mM), molybdate (K_i = 0.055 mM) and pyrophosphate (K_i = 6.7 mM) and non-competitively by fluoride (K_i = 9.8 mM). Metal ions such as Hg^{2+}, Cu^{2+} and Zn^{2+} express an inhibitory effect on the enzyme, while the enzyme is slightly activated by non-ionic detergents, Tween 20 and Triton X-100. There is no change in the enzyme activity in the presence of tartrate, citrate, EDTA, 1,10-phenanthroline and sulfhydryl-group modifiers such as *p*-chloromercuribenzoate and *N*-ethylmaleimide.

Keywords: acid phosphatase, *Artemisia vulgaris*, Compositae, mugwort, pollen, purification.

INRODUCTION

Plant AcPases are present in a variety of plant tissues and frequently occur in multiple forms which differ in molecular mass,[1] pI value,[2] substrate specificity[3] or carbohydrate content.[4] In general, they occur in very small quantities in plant tissues, are unstable in dilute solutions, and are subject to surface denaturation in the pure state. Although these properties often make the isolation of acid phosphatases difficult, a number of plant AcPase enzymes have been isolated and characterised from different plant tissues, like roots,[2] seeds,[5] tubers[6] or maize scutellum.[7] Their function is not exactly known, but it is likely that they participate in the mobilisation of inorganic phosphorous (roots, seeds).

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Many authors have detected the following enzymes in pollen grains: phosphatases, proteases, glucidases and lipases.[8] In the limited findings concerning the acid phosphatase activity of different pollen extracts of grasses, weeds and trees, it was established that AcPases are located in the intine of the pollen wall and, with other enzymes, they make up a large portion of the mobile (soluble) protein in pollen grain. The biological role of these enzymes is believed to be connected with germination, early pollen tube nutrition and penetration of the stigma surfaces.[9]

In this paper, the isolation, partial purification and characterization of an acid phosphatase from a pollen extract from mugwort (*Artemisia vulgaris*) are reported. This particular weed belongs to family Compositae, widely distributed in Europe, and represents a common cause of hay fever during late summer.[10] Until now there have been no reports concerning the isolation and characterization of this kind of enzyme from an allergenic pollen extract.

RESULTS AND DISCUSSION

The presence of multiple forms of acid phosphatases in the pollen extract of *Artemisia vulgaris* was shown by analytical IEF (Fig. 1). The most acidic form (the most active towards α-NP) named Acp-1 was separated by ion-exchange chromatography on CM Sephadex C-50, additionally purified by DEAE Sephadex A-50 column chromatography and Sephadex G-100 sf gel filtration (Fig. 2). After the final step, the enzyme had been purified 48-fold over the crude extract with an overall yield of 2.6 %. The isolated enzyme

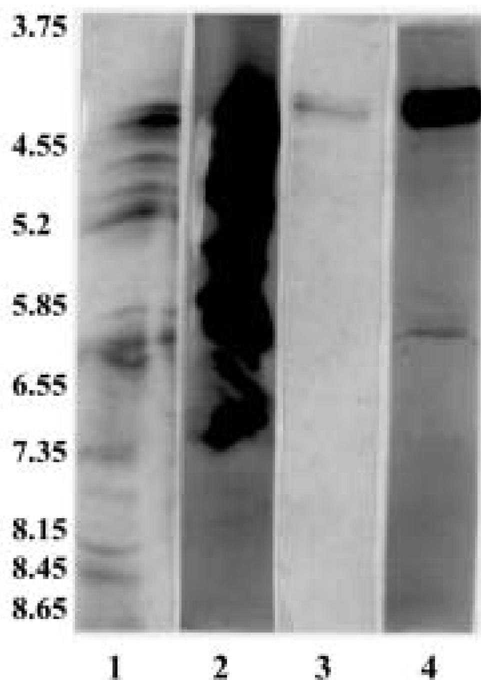

Fig. 1. Isoelectrofocusing and activity staining of mugwort pollen extract proteins and the purified enzyme. Lane 1. mugwort pollen extract proteins, Coomassie Brilliant Blue R-250 (CBB) stained, 12.5 μg of proteins; lane 2. Acp-1, 2.6 μg of proteins, CBB stained; lane 3. mugwort pollen extract acid phosphatases detected with α-naphthyl phosphate/Fast Blue RR salt, 12.5 μg of proteins; and lane 4. Acp-1, 2.6 μg, activity staining with α-naphthyl phosphate/Fast Blue RR salt.

makes up about 0.05 % of the total pollen extract proteins. The relatively low purification factor achieved during the early stages of the enzyme purification may be attributed to the presence of multiple forms of acid phosphatases in the extract of *A. vulgaris* pollen, but also to the instability of the enzyme.

The M_r of Acp-1 by gel filtration through Sephadex G-100 sf was determined to be 76 kDa. The purified enzyme was homogeneous on SDS polyacrylamide gel electrophoresis. In analytical isoelectric focusing, the enzyme appears as two sharp and very close bands, with a pI at 4.2 (Fig. 1). The effect of pH on the enzyme activity was assayed at different pH values in Gly-HCl buffer, acetate buffer and Tris-maleate buffer over the pH range of 2.5 – 8.5. The maximum activity of this phosphatase was found to be at pH 5.4 (with half maximum activities at pH 3.8 and 7.1). The highest activity for *p*-nitrophenyl phosphate (*p*-NPP) hydrolysis was obtained at 50 ºC, but thermal stability studies indicated that above this temperature the enzyme becomes unstable losing 50 % of its activity after 5 min at 70 ºC. The relative rates of hydrolysis of various substrates (Table I) indicate that the acid phosphatase obtained from the extract from mugwort pollen dephosphorylates a wide variety of phosphate esters.

The kinetic parameters for *p*-NPP hydrolysis, K_m and V_{max} were determined to be 0.16 mM and 18.1 U/mg of protein. Similar K_m values of other plant acid phosphatases were found to be in the range of 0.07 mM[11] – 13.25 mM.[12]

The *A. vulgaris* pollen extract phosphatase was competitively inhibited by inorganic phosphate with a K_i value of 1.25 mM. This inhibition could play a physiological role in sustaining a stable level of phosphate during pollen tube growth on the basis of a feedback regulation.

TABLE I. Substrate specificity of mugwort pollen extract acid phosphatase. The relative rates for the hydrolysis of various phosphate esters are expressed as a per cent of the activity level with 4-NPP as the substrate

Substrate	Relative activity (% of 4 - NPP)
4-NPP	100
Pyrophosphate	140
α-NP*	47
Glucose-6-P	36
O-Phospho-L-threonine	52
O-Phospho-L-serine	30
cAMP	22
O-Phospho-L-tyrosine	88
Glucose-1-P	6
Fructore-1,6-P_2	33
ATP	22

*4 mM substrate concentration

The enzyme was inhibited competitively by molybdate, a transition state analogue, with a K_i of 0.055 mM, and non-competitively by NaF with a K_i of 9.8 mM. These values

are within the range typical for other plant acid phosphatases. Pyrophosphate also competitively inhibits *p*-NPP hydrolysis with a K_i of 6.7 mM.

The majority of properties of *A. vulgaris* acid phosphatase resemble those of other plant phosphatases. According to the obtained results, the most convenient substrates for this acid phosphatase, apart from *p*-NPP, were pyrophosphate (PPi) and *O*-phospho-L-tyrosine. Efforts to separate pyrophosphatase and *p*-NPP activities by gel-chromatography failed (Fig. 2), which favours the idea that these activities in the isolated enzyme preparation are associated with the same or very similar types of proteins. Many authors have reported significant pyrophosphatase activity of acid phosphatase.[1–3,11,13–15]

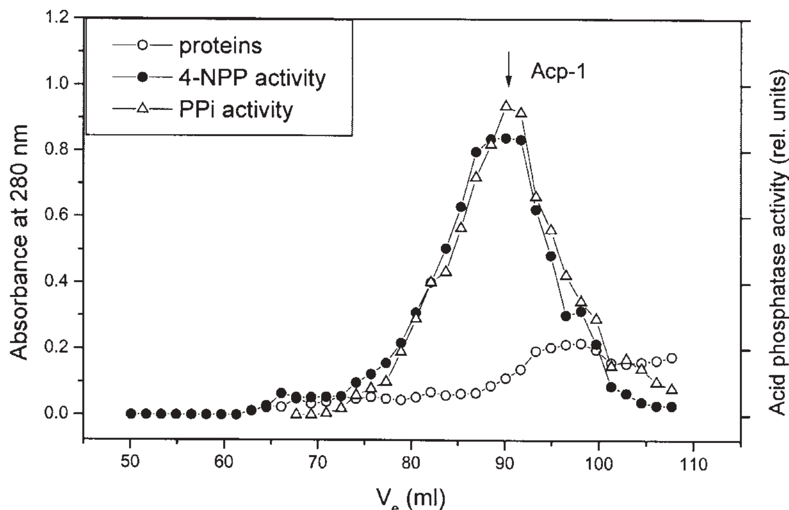

Fig. 2. Purification profile of mugwort pollen extract acid phosphatase and pyrophosphatase activities through Sephadex G-100 sf column chromatography.

Pyrophosphate represents a by-product of DNA and protein biosynthesis and by breaking down PPi this enzyme shifts the equilibrium of these reactions towards synthesis. As growing cells produce high levels of pyrophosphate during the pollen tube growth, an acid phosphatase from a soluble part of pollen grain may contribute to the pollen tube growth, germination and nutrition by liberating phosphate from a variety of different phosphate ester compounds and especially from inorganic pyrophosphate.

EXPERIMENTAL

Pollen source and extract preparation.

The pollen samples were obtained from the Institute for Immunology and Virology, Torlak, Belgrade, Yugoslavia. Dry (defatted by ethyl ether) pollen (30 g) was suspended in 300 ml distilled water and gently shaken for 12 h at 4 ºC. The pollen extract, obtained by centrifugation was stored at 4 ºC.

Protein content and enzyme determination.

The protein content was determined according to Bradford.[16] All enzymatic assays during the purification procedure were performed in 0.1 M sodium acetate buffer (pH 5.0) with 6 mM *p*-NPP as the substrate (Sigma

Chemical Co.). The phosphatase activity was measured by mixing 10 μl of the sample with 100 μl of substrate solution. After 5 min of incubation at 30 ºC, the reaction was stopped by addition of 50 μl 3 M NaOH. The AcPase activity was measured at 405 nm by monitoring the release of *p*-nitrophenol (*p*-NP), using a molar extinction coefficient of 18,000 M^{-1} cm^{-1}. One unit (U) of enzyme is that required to release 1 μmol of *p*-NP per min.

Purification of the acid phosphatase.

All operations were carried out at 4 ºC. AcPase activity during all stages of purification was monitored using 6 mM *p*-NPP as the substrate.

1. The pollen extract was dialysed against 20 mM acetate buffer pH 5.4 (buffer A). The dialysate was centrifuged at 3000 rpm for 15 min and then poured onto a CM Sephadex C-50 column (2 × 10 cm), equilibrated with buffer A. The active fractions, eluted as unbound proteins, were pooled, concentrated by ultrafiltration and dialysed over night against 20 mM Tris buffer pH 7.5 (buffer B).

2. The dialysate was centrifuged at 3000 rpm for 15 min and then poured onto a DEAE Sephadex A-50 column (2 × 10 cm) column, equilibrated with buffer B. The column was eluted at 12 ml/h with a linear NaCl gradient (0 – 0.5 M NaCl in buffer B).

3. The partially purified extract was then loaded onto a 0.75 × 90 cm Sephadex G-100 sf column pre-equilibrated with 0.9 % NaCl. The proteins were eluted with the same buffer at a flow rate of 12 ml h^{-1} and 3 ml fractions were collected. The fractions with the highest specific activity were concentrated rapidly.

Assay of the enzymatic activity.

Assays to determine the pH optimum were carried out with 0.1 M NaOAc buffer in pH range of 3.6–5.6 and Tris-maleate buffer in pH range of 5.5–8.5. The effect of metal ions, inhibitors and modifiers (1 mM) was assayed with 6 mM 4-NPP in 0.1 M NaOAc buffer pH 5.4. The thermal stability of the phosphatase activity of the pollen samples was investigated by incubation of pollen extracts at 40 ºC, 50 ºC, 60 ºC and 70 ºC. Aliquots were taken every 10 min during 1 h and immediately placed in an ice-bath for 1 h before measuring the residual phosphatase activity under the conditions described above.

The substrate specificity studies were carried out at 30 ºC for 15 min by determining the inorganic phosphate according to Lowry and Lopez.[17] The incubation medium contained 2 mM (or 4 mM) phosphoric ester in 0.1 M sodium acetate buffer (pH 5.4).

The inhibition constant (K_i) of some inhibitors with respect to *p*-NPP hydrolysis was detemined graphically using the Lineweaver-Burk plot and by a replot of the slopes against the concentration of the inhibitor.

Polyacrylamide gel electrophoresis (PAGE).

Subunit analysis was carried out by SDS-PAGE performed in a vertical slab gel 4/10 %, according to Laemmli[18] in a Hoefer apparatus. The analytical isoelectrofocusing was performed as described previously.[19]

The enzyme activity was detected by incubating the gel in a solution made with 1 mg/ml each of α-naphthyl phosphate/Fast Blue RR salt in 0.1 M sodium acetate buffer pH 5.4.

CONCLUSION

The major isoform of *Artemisia vulgaris* pollen extract acid phosphatase has been purified and partly characterised. The majority of properties of *A. vulgaris* acid phosphatase resemble those of other plant phosphatases. The purified enzyme has broad specificity, and from the potential *in vivo* substrates, pyrophosphate and *O*-phospho-L-tyrosine are estimated to be the best substrates for this enzyme. The enzyme is competitively inhibited by phosphate, molybdate and pyrophosphate and non-competitively by fluoride. The inhibition by phosphate and pyrophosphate could play a possible physiological role during the germination of the pollen tube. The results shown in this study could also help in the elucidation of the pathogenesis of the allergic reaction.

ИЗВОД

ИЗОЛОВАЊЕ И ДЕЛИМИЧНА КАРАКТЕРИЗАЦИЈА КИСЕЛЕ ФОСФАТАЗЕ ЕКСТРАКТА ПОЛЕНА *Artemisia vulgaris*

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Кисела фосфатаза екстракта полена високог корова (*Artemisia vulgaris)* је пречишћена 48 пута комбинацијом јоноизмењивачке и гел-хроматографије. Молекулска тежина ензима је 76 kDa и 73 kDa, одређена гел-филтрацијом на матриксу Sephadex G-100 sf и SDS PAG електрофорезом (при редукујућим и нередукујућим условима), респективно. При изоелектрофокусирању, ензим се састоји из две врло блиске траке pI вредности око 4,2. Оптимално pH за активност ензима је 5,4. Привидно K_m за хидролизу *p*-нитрофенил-фосфата је процењено да је 0,16 mM. Пречишћени ензим има широку специфичност, хидролизује *p*-нитрофенил-фосфат и α-нафтил-фосфат. Пирофосфат и *O*-фосфо-L-тирозин су процењени као најбољи од потенцијалних *in vivo* супстрата овог ензима. Ензим је инхибиран компетитивно фосфатом (K_i = 1,25 mM), молибдатом (K_i = 0,055 mM) и пирофосфатом (K_i = 6,7 mM) а некомпетитивно флуоридом (K_i = 9,8 mM). Јони метала, као што су Hg^{2+}, Cu^{2+} и Zn^{2+} исказују инхибиторни ефекат на ензим, док је ефекат не-јонских детергената, као што су Tween 20 и Triton X-100 благо активирајући. Нема промене у активности ензима у присуству тартарата, цитрата, EDTA, 1,10-фенантролина и модификатора сулфхидрилних група као што су *p*-хлоромеркурибензоат и *N*-етилмалеимид.

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REFERENCES

1. T. K. Biswas, C. Cundiff, *Phytochemistry* **30** (1991) 2119
2. F. Panara, S. Pasqualini, M. Antonielli, *Biochim. Biophys. Acta.* **1037** (1990) 73
3. C. V. Ferreira, J. M. Granjeiro, E. M. Taga, H. Aoyama, *Biochem. Biophys. Res. Commun.* **242** (1998) 282
4. I. Lorenc-Kubis, *Plant Sci.* **44** (1986) 1
5. M. Olczak, W. Watorek, B. Morawiecka, *Biochim. Biophys. Acta.* **1341** (1997) 14
6. K. Gellatly, G. B. B. Moorhead, S. M. G. Duff, D. D. Lefebvre, W. C. Plaxton, *Plant Physiol.* **106** (1994) 223
7. A. Rossi, M. S. Palma, F. A. Leone, M. A. Brigliador, *Phytochemistry* **20** (1981) 1823
8. R. G. Stanley, H. F. Linskens, "Enzymes and Cofactors", in *Pollen Biology Biochemistry and Management*, Springer-Verlag, New York, 1974, p. 193
9. R. B. Knox, Y. Heslop-Harrison, *J. Cell Sci.* **6** (1970) 1
10. G. C. Blanchard, R. Gardner, *Ann. Allergy* **36** (1976) 40
11. H. J. A. Ullah, D. M. Gibson, *Arch. Biochem. Biophys.* **260** (1988) 514
12. R. A. Penheiter, M. G. S. Duff, G. Sarath, *Plant Physiol.* **114** (1997) 597
13. M. Ferens, B. Morawiecka, *Phytochemistry* **24** (1985) 2839
14. S. T. Kaneko, R. Kikuti, K. Kubota, *Phytochemistry* **29** (1990) 2883
15. P. P. Waymack, L. R. Van Etten, *Arch. Biochem. Biophys.* **288** (1991) 621
16. M. Bradford, *Anal. Biochem.* **72** (1976) 248
17. O. H. Lowry, J. A. Lopez, *J. Biol. Chem.* **162** (1946) 421
18. U. K. Laemmli, *Nature* **227** (1970) 680
19. T. D. Ćirković, M. N. Bukilica, M. Đ. Gavrović, Z. M. Vujčić, S. Petrović, R. M. Jankov, *Allergy* **54** (1999) 128.