BIOLOGIA PLANTARUM (PRAHA)

29 (6) : 401–408, 1987

Some Immunochemical Properties of Pollen Extracellular Nuclease

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Abstract. The extracellular nuclease from *Pinus silvestris* pollen was isolated and characterized. Molecular sieving produced only one peak of nuclease with apparent molecular mass of about 30 kDaltons, while molecular mass of the enzyme as determined by means of sodium dodecyl sulphate-DNA gel electrophoresis was 25.7 kDaltons or less. The nuclease exhibited one electrophoretic molecular form only.

Specific antiserum against this nuclease was prepared and the nuclease was shown to be immunochemically cross-reactive with extracellular nuclease from *Pinus nigra* pollen. No immunochemical relationships were observed between pine nuclease and the pollen extracellular nucleases from the angiosperm species, *Nicotiana tabacum*, *Corylus avellana*, *Alnus incana* and *Betula pubescens*. No cross-reactivity was detected by double diffusion between the pine nuclease and the extracellular proteins from vegetative spores of *Equisetum arvense*.

In our previous work, nuclease was demonstrated to be released by various pollens from phylogenetically more or less remote plant species (MATOUŠEK and TUPÝ 1985, MATOUŠEK 1986). The tobacco pollen enzyme was identified as a sugar non-specific nuclease (E.C.3.1.30.x) (MATOUŠEK and TUPÝ 1984). A similar enzyme was detected in diffusate from pollen of *P. nigra* (MATOUŠEK and TUPÝ 1985). Nucleases from various pollen species exhibit similar substrate specificities (MATOUŠEK 1986), suggesting that some catalytical properties of the enzymes are evolutionarily conservative. On the other hand, nothing is known about immunochemical specificity of pollen extracellular nucleases.

In the present work, the possible similarity of the pollen nucleases is examined by immunochemical methods.

MATERIAL AND METHODS

Plant Material

Vegetative spores were collected from *Equisetum arvense* L. Pollen was collected from the following plant species grown under natural or field con-

Received April 13, 1987; accepted June 16, 1987

ditions: *Pinus silvestris* L., *Pinus nigra* ARNOLD, *Alnus incana* L., *Betula pubescens* EHRH., *Corylus avellana* L. and *Nicotiana tabacum* L. Pollen and the spores were stored at −25 °C until used.

Preparation of Diffusates and Crude Extracts

Pollen difusates were obtained by shaking 50 mg of pollen or spores for 3 min in 1 ml of 10 % sucrose solution and then by filtering the suspensions at slightly reduced pressure. Unless stated otherwise, the proteins of the diffusates were precipitated by $(NH_4)_2SO_4$ at 85 % saturation for 1 h in an ice cold water bath, sedimented for 15 min at 19 000 *g* and dissolved in 10 % sucrose. The samples obtained by this procedure are termed "crude extracts".

Purification of Extracellular Nuclease from *P. silvestris* Pollen

The nuclease of pollen diffusate obtained from 25 g of pollen was recovered by precipitation with 50—85 % $(NH_4)_2SO_4$ for 1 h. All steps of nuclease purification were made in cold (0 °C).

Proteins precipitated at 50 % $(NH_4)_2SO_4$ were removed by centrifugation for 30 min at 27 000 *g*. The $(NH_4)_2SO_4$ concentration was then adjusted to 85 % and 1/3 V/V of absolute ether was added to the suspension in order to remove lipoidal and oil impurities by shaking the suspension vigorously for 2 min. Then the suspension was centrifuged for 30 min at 27 000 *g* and the pellet was dissolved in 1 ml of 0.2 M acetate buffer pH 4.8 containing 1 mM $ZnSO_4$ and 1 mM $MgSO_4$ (starting buffer). Zn^{2+} and Mg^{2+} ions increased the stability of the nuclease (unpublished results). The sample was centrifuged in cold at 8500 *g* for 10 min in order to remove insoluble proteins and impurities. The solution was then dialyzed against 300 ml of the starting buffer for 2 h and the proteins were fractionated by gel filtration on Sephadex G-75 (fine) column under conditions described by MATOUŠEK and TUPÝ (1984). Fractions 30—38 (Fig. 2), containing essentially all of the nuclease activity were pooled and concentrated with polyethylene glycol 20 000 to 1.2 ml. Finally, 0.4 ml of 100 % glycerine was added. The sample, which contained 160 μg of protein, was aliquoted and stored at −25 °C until used for immunization and further analysis.

Assay of Nuclease Activity

Nuclease activity with highly polymerized native calf thymus DNA (Serva, Heidelberg) was determined by following the production of acid soluble (2 % $HClO_4$) material over a 90 min period and calculated from a three point assay (MATOUŠEK 1986). Optimal pHs of ACB were used for nuclease activity estimations, pH 4.8 for pine nuclease and pH 5.2 for the other pollen nucleases (MATOUŠEK and TUPÝ 1985). In individual fractions obtained by gel filtration on Sephadex G-75, nuclease activity was detected by means of radial diffusion method using agarose containing native calf thymus DNA (MATOUŠEK *et al.* 1987).

Preparation of Antiserum

A 6-month-old male hybrid rabbit was immunized with a total of 145 μg of purified pine pollen extracellular nuclease containing 50 669 U of DNAse

Abbreviations used: PBS = 0.01 M phosphate buffer pH 7.2, 0.15 M NaCl; ACB = 0.2 M acetate buffer of indicating pH, 1 mM $ZnSO_4$; Mr = molecular mass.

activity. The rabbit was injected each time subcutaneously on the back at several sites with 385 μl of antigen emulsified with an equal volume of Freund's complete adjuvant. Three injections were given at one week intervals. 1/10 V/V of tenfold concentrated physiological saline was added to the antigen solution just before immunization. A booster injection was given 3 weeks after the third priming immunization, with 45 μg of the antigen in saline solution without Freund's adjuvant. The rabbit was exsanguinated two weeks after the boost. Serum was lyophilized and stored at 4 °C.

Preparation of Immunosorbent and Immunochemical Tests

The serum IgG fraction was purified by ammonium sulphate precipitation of immunoglobulins at 50 % saturation followed by fractionation on a DEAE-TRISACRYL column (CORTHIER *et al.* 1984). Immunosorbent (IgG linked to beaded cellulose by diazotization of the 4-aminophenylsulfonylethyl derivate) was prepared as described by TURKOVÁ *et al.* (1986) and kindly provided by M. Beneš (Inst. Macromolecular Chemistry, Czechoslovak Acad. Sci., Praha). A total of 2.28 mg of protein (IgG fraction) was bound to 1 g of wet cellulose. Immunosorbent was resuspended in PBS at a concentration of 600 mg ml^{-1}, stabilized with sodium azide and stored at 2 °C.

For immunotitrations, aliquots (25, 50, 75 and 150 μl) of the immunosorbent were pipetted into the Eppendorf tubes and centrifuged for 1 min at 3000 *g*. In each case, immunosorbent was then resuspended in 0.7 ml of 0.02 M Tris-borate buffer pH 7.4 containing 0.15 M NaCl and incubated overnight at 6 °C with 100 μl of the crude extracts of the nucleases, mixing the contents of each tube three times during this period. After sedimentation for 1 min at 3000 *g* nuclease activity was determined in 100 μl aliquots of the supernatants. Nonimmune IgGs bound to cellulose, inactivated cellulose alone and the samples of the nucleases without immunosorbent were used as controls.

Double diffusion was performed as described by OUCHTERLONY (1949). Reactions were carried out in agarose-starch gels buffered with borate-phosphate buffer pH 7.6 (*e.g.* KLOZ and KLOZOVÁ 1974).

Activity Gel Electrophoresis

Activity gel electrophoresis on SDS-DNA gels was performed as described by FRASER *et al.* (1986) with following modifications: Sample buffer and the gels were prepared without bovine serum albumin. The 9.5 % gels contained 18.5 μg ml^{-1} of native DNA and were polymerized in 12 cm long rods, 57 mm in diameter. Running gel and spacer gel were 9 and 2 cm long, respectively. The electrophoresis was carried out for 10 min at a curent of 1 mA and subsequently at 3.5 mA per gel. After renaturation step, the gels were incubated in ACB pH 4.8 at room temperature for 10 h in order to detect nuclease activity.

The gels were stained for proteins using Coomassie blue R250 and were scanned using Soft Laser Scanning Densitometer SL-TRFF (Biomed Instruments, Inc.). The following prestained (Coomassie blue) protein standards were simultaneously run as molecular mass markers: myosin (H-chain), phosphorylase B, bovine serum albumin, ovalbumin and α-chymotrypsinogen, having 200, 92.5, 68, 43 and 25.7 kDaltons, respectively.

Other Methods

Native gel electrophoresis was performed as has been already described by MATOUŠEK and TUPÝ (1985). Proteins were quantified by the method of BRADFORD (1976).

RESULTS

Purification and Characterization of Extracellular Nuclease from *P. silvestris* Pollen

The finding that pine pollen releases comparatively high amount of nuclease exhibiting high specific activity (MATOUŠEK and TUPÝ 1985) facilitated the overall purification of the enzyme. As can be seen in Fig. 1, pine pollen diffusate contained high amounts of large proteins (having Mr > 60 kDaltons), which can be simply separated from pollen nuclease by gel filtration through Sephadex G-75 since the nuclease has a native molecular mass of about 30 kDaltons (Fig. 2). In this simple procedure of purification (Table 1), a relatively high specific activity of nuclease (349 400 U mg^{-1} protein) was achieved. Only one band of protein was revealed in final sample by SDS gel electrophoresis and Coomassie blue staining procedure (Fig. 3, Channel 1). Because the proteins, as well as α-chymotrypsinogen (Mr = 25.7 kDaltons) comigrated with tracking dye (bromphenol blue), their molecular mass was 25.7 kDaltons or less. Nuclease activity was detected as the protein on

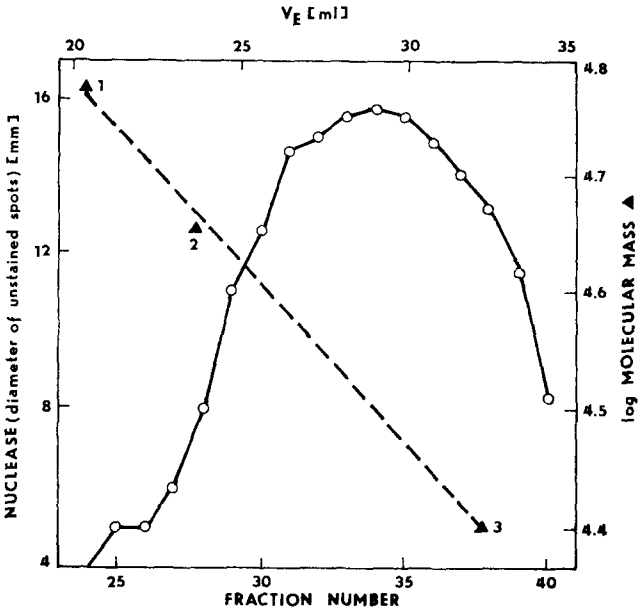

Fig. 2. Molecular sieving of pine pollen extracellular nuclease. 1 ml of crude extract containing 79 600 U of DNAse activity and 1.14 mg of protein was loaded on a Sephadex G-75 (fine) column, equilibrated with 0.2 M acetate buffer pH 4.8; 1 mM $ZnSO_4$; 1 mM $MgSO_4$. Nuclease activity was detected in 10 μl aliquots of individual fractions by radial diffusion method (see Material and Methods) and is represented by diameter of unstained spots in millimetres. The numbers 1,2 and 3 refer to the positions of elution of glucose oxidase, ovalbumin and chymotrypsin A with molecular masses of 60, 45 and 25.3 kDaltons, respectively.

TABLE 1

Purification of nuclease by ammonium sulphate fractionation and gel filtration on Sephadex G-75. Activity with native DNA is expressed in units from the original amount of 25 g of pollen

	Total nuclease activity [U]	Amount of proteins [mg]	Specific activity [U mg^{-1} protein]
Extracellular proteins of pollen diffusate	87 500	7.50	11 666
Fraction of acid soluble proteins precipitated by $(NH_4)_2SO_4$ at 50–85 % saturation and dialysed	79 600	1.14	69 824
Sephadex G-75 fractions 30 to 38	55 911	0.16	349 443

the activity gel (compare Channel 1 and Channel 2 in Fig. 3), corresponding to Mr $\leq$ 25.7 kDaltons. Electrophoretic analysis of nuclease revealed one molecular form of the enzyme only (Fig. 4). This result along with the results obtained by molecular sieving (Fig. 2) and SDS gel electrophoresis (Fig. 3) favour the absence of multiple molecular forms of nuclease from *P. silvestris* pollen.

Immunochemical Properties of Pollen Extracellular Nuclease

From the results in Fig. 5 it can be concluded that specific antibody was obtained against extracellular nuclease from *P. silvestris* pollen. Incubation of 29 U of DNAse activity of the nuclease with 150 µl of immunosorbent (*i.e.* with 205.2 µg of immobilized proteins of IgG fraction) led to the absorption of practically the entire amount of enzyme, whereas no immunosorption occurred when nonimmune (control) serum was bound to cellulose or when inactivated cellulose was used (data not shown). Quantitatively similar immunosorption was also obtained with the *P. nigra* nuclease (Fig. 5). On the other hand, no absorption of extracellular nucleases from *N. tabacum* and *C. avellana* pollen occurred (Fig. 5), indicating that the antigenic determinants on these two pollen nucleases are not immunochemically related to antigenic determinant(s) on the pine pollen nucleases studied. The results (data not shown) were confirmed in an immunotitration test using Pansorbin as immunosorbent (the method see *e.g.* FRASER *et al.* 1986) and by double diffusion (Fig. 6). In the latter test, immunoprecipitation was also detected only with protein from *P. silvestris* or *P. nigra* pollen diffusates (Fig. 6, plate a — 3,6 and plate b — 3, 5, 6). No immunoprecipitation was observed with crude extracts from *N. tabacum*, *C. avellana*, *B. pubescens* and *A. incana* pollen (Fig. 6). In addition, no obvious immunochemical reaction occurred between the protein(s) in the purified sample of the nuclease from pine pollen and diffusible proteins from horsetail (*E. arvense*) vegetative spores (Fig. 6, plate a — 5). Neither DNAse nor RNAse activity was detected in diffusate from *E. arvense* spores.

DISCUSSION

Extracellular nuclease from *P. silvestris* pollen had an apparent Mr = 25.7 kDaltons or less after denaturation, while its native molecular mass was estimated to be about 30 kDaltons (compare Figs. 2 and 3). Such a difference in the molecular masses of the enzyme could be due to the presence of subunits

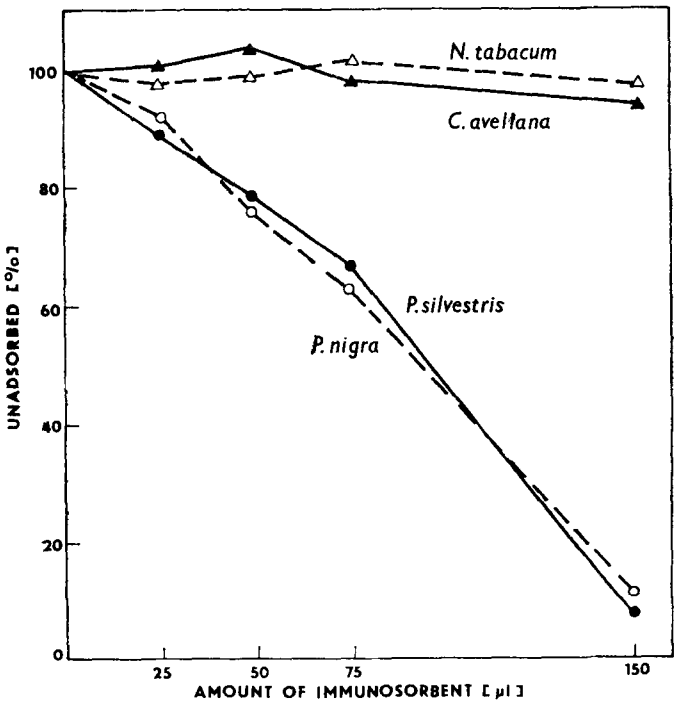

Fig. 5. Immunotitration of the pollen extracellular nucleases using immunosorbent. — 100 µl of the crude extracts were incubated with the immunosorbent described in Material and Methods. The units corresponding to 100 % of relative activity applied in each case were respectively for *P. silvestris*, 29 U; *P. nigra*, 30 U; *N. tabacum*, 27 U; *C. avellana*, 25 U. The specific nuclease activities applied were for *P. silvestris*, *P. nigra*, *N. tabacum* and *C. avellana* respectively 13 895, 18 850, 6080 and 2031 U mg^{-1} protein.

separable by denaturation procedure and/or post-translational modification of the enzyme (*e.g.* glycosylation). The presence of two subunits (15 and 25 kDaltons) separable by reduction of disulfide bonds was found in plant nuclease I from *Phaseolus aureus* L. (KOWALSKI *et al.* 1976). The glycoprotein nature of plant nuclease I was described by KOWALSKI *et al.* (1976) and by CLAMPHAM (1980). According to our unpublished results, a lower molecular mass after denaturation as opposed to native Mr (25—33 kDaltons) is also true for molecular forms of nuclease from tobacco pollen characterized earlier (MATOUŠEK and TUPÝ 1984). It is known that the relative molecular masses of plant nucleases (presumably nuclease I) vary considerably, depending on a plant, organ and tissue source (for reviews see WILSON 1982 and FARKAS 1982).

Only one molecular form and absence of enzyme polymorphism was found for nuclease from *P. silvestris* pollen (Figs. 2, 3 and 4). One electrophoretic form of nuclease having a relative molecular mass of about 29.5 kDaltons was also detected earlier in protein diffusate from *P. nigra* pollen (MATOUŠEK and TUPÝ 1985).

The specific antiserum against pine extracellular nuclease was relatively easily obtained (Fig. 5). Antibody against plant nuclease I from *Tradescantia* leaves was obtained by CLAMPHAM (1980), but no information is available about possible immunochemical cross-reactions with other plant nucleases. Although the catalytic properties of pollen extracellular nucleases from different phylogenetically more or less remote plant species are very similar (MATOUŠEK and TUPÝ 1985, MATOUŠEK 1986), immunochemical cross-reactions were only detected between the extracellular nucleases from *P. nigra* and *P. silvestris* (Fig. 5). No immunochemical relationships were observed in our experiments between pine nuclease and the nucleases of the angiosperm species investigated (Figs. 5 and 6), suggesting that pollen extracellular nucleases are rather immunochemically specific. At last genera-related immunospecificity was revealed for pinus pollen nuclease. It is not known whether the immunospecificity of the pollen enzyme is due to differences in their primary protein structures (and the corresponding genes) or due to differences in post-translational modification(s) of the nucleases. It is of interest to mention here the fact that the extracellular nuclease from *P. silvestris* pollen is also not immunochemically cross-reactive with *Neurospora* endo-exonuclease and the two phosphate-repressible nucleases (A and B) of *Neurospora* (M. J. Fraser, personal communication). The *Neurospora* endo-exonuclease was previously shown not to be immunochemically cross-reactive with mung bean nuclease (plant nuclease I) (FRASER *et al.* 1986).

No nuclease activity was detected in the aqueous diffusate from *E. arvense* vegetative spores. Moreover, no immunochemically related proteins(s) to pine nuclease were revealed in this diffusate (Fig. 6). These results provide, along with the results described earlier (MATOUŠEK 1986), additional evidence for the hypothesis (MATOUŠEK 1986, MATOUŠEK and TUPÝ, in press) that pollen extracellular nucleases first appeared in evolution in relation to physiological and biochemical adaptation of male gametophyte on sporophyte.

Acknowledgements

The authors would like to thank Murray J. Fraser (Department of Biochemistry, McGill University Montreal, Canada) and Eva Klozová (Department of Genetics, Institute of Experimental Botany, Czechoslovak Academy of Sciences) for their fruitful discussions and help in the preparation of this manuscript.

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Figs. 1, 3, 4 and 6 at the end of the issue.

BOOK REVIEW

HORGAN, R., JEFFCOAT, B. (ed.): CYTOKININS: PLANT HORMONES IN SEARCH OF A ROLE. — BPGRG Bristol 1987. Monograph 14, 113 pp.

This book contains proceedings of a meeting organized by The British Plant Growth Regulator Group on 8th May 1986 at The SCI Lecture Theatre, Belgrave Square in London. Eight contributions written by 13 authors deal with topics of cytokinin research.

The results of cytokinin biosynthesis study in crown gall tissues are presented by R. Horgan. The contribution of B. A. McGaw brings evidence that O-glucosyl conjugates may be cytokinin storage forms, and N-glucosyl and N-alanyl conjugation and N^6-side chain cleavage are irreversible processes. The possible role of 5′-nucleotidase, adenosine nucleosidase and adenine phosphoribosyltransferase in the control of cytokinin metabolism and interactions between the metabolism of cytokinins and unsubstituted purines are discussed by T. Stuchbury and L. R. Burch. Two modern methods of cytokinin measurements (stable isotope dilution analysis and immunological assays) are described in detail by T. L. Wang and R. Horgan. T. L. Wang reports on work on *Phaseolus* and *Physcomitrella patens* (moss) cytokinin mutant systems. Undesirable side-effects and positive ones of applied cytokinins are discussed in relation to their potential practical applications in horticulture and agriculture by T. H. Thomas and D. Blakesley. The hypothesis that cytokinins, after having been transported through an apoplastic pathway from the sites of synthesis, affect the water balance of whole plants by acting on the leaf to open stomata is examined by L. D. Incoll and P. C. Jewer. The role of cytokinins in initiating sink activity during the early stages of grain growth was studied by J. R. Lenton and N.E.J. Appleford.

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