Antitumor and biological effects of black pine *(pinus nigra)* pollen nuclease

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The antitumor effect of black pine *(Pinus nigra)* pollen nuclease (PN) tested *in vitro* was negligible in comparison with bovine seminal ribonuclease (BS-RNase). However, in the experiments *in vivo* a significant decrease of the human melanoma tumor size was observed in the mice treated with this nuclease and also with the animal RNases and DNase I. In nude mice injected intratumoraly with PN (10μg/dose) the tumor size decreased from 100% in the control mice to 46% in treated mice whereas in counterparts treated with BS-RNase and DNase I the tumor growth was reduced a little more, however after ten times higher doses (100 and 80μg per dose). Certain aspermatogenic and embryotoxic activity as an expression of side effects of PN and comparative enzymes also appeared, but it was lower compared to the effect of bovine seminal ribonuclease. Immunogenicity of PN was significantly weaker in compaison with BS-RNase.The antibodies against black pine nuclease produced in the injected mice did not inactivate the biological effects of this plant nuclease *in vivo.* In conclusion PN nuclease proved *in vivo* higher antitumor activity against human melanoma tumors growing in athymic mice in comparison with animal bovine seminal ribonuclease and DNase I.

Keywords: antiproliferation, cell, immunogenicity, melanoma, mice, nuclease

Although many plant nucleases have been studied in relation to biological processes in plants [1,3-11], there is only one information available about possible biologic activity of these enzymes on mammalian cells and tissues: A homologue of nuclease I, mung bean sprout nuclease, was shown in our recent work to have an anti-tumorogenic effects in animals [2]. An important property of the nuclease I group (to which many nucleases belong) is the particular specificity towards secondary structures of nucleic acids and its ability to cleave different homopolymers. Despite many observations suggesting that nuclease I group is involved in many processes, which are strongly regulated during development and influenced by many factors such as light, phytohormones, wounding, water stress and infection with some plant pathogens [3,4] the more exact analysis of nuclease I expression was obtained for its participation in processes of apoptosis and plant senescence [6-12].

Extracellular pollen nuclease was characterized in our previous work to be an enzyme of the nuclease I type, that is similar to fungal S-nuclease, except of much lower preferences for single-stranded substrates [23, 25]. Binucleate pine (*Pinus nigra*) pollen has been used to isolate plant nuclease with high specific activity [26] and therefore, it could serve as a very pure source of plant nucleolytic enzymes for biotechnology, as well as for biological experiments to investigate potential antitumor activity of plant nucleases.

In addition to extracelular character of pollen nucleases, there are some methodical advantages for studies of nuclease I expression and function(s), as well as for possible biotechnology experiments. Unlike to specific RNases or DNases, nuclease can be easily identified in activity gels (containing or sorbing nucleic acids, various RNA or DNA substrates) owing to its dual character of activity. Both systems, on native, or on denaturing (with re-naturation) gels [23, 26, 33] are available. In addition to classical absorbance or radial diffusion [23, 24,

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26] activity assays, it is possible to use supercoiled DNA substrates for supplementary, but very sensitive method [25]. Nuclease I purification procedures are also described in various papers; it usually includes several steps such as precipitation with 80-85% ammonium sulfate and column chromatography on Sephadex gels, ion-exchange chromatography (e.g. on DEAE Agarose) followed by affinity chromatography [32].

S-like plant nucleases I have similar molecular weight and structure as animal ribonucleases [13-22]. While bovine pancreatic RNase A exerts a minor antitumor effect, BS-RNase exerts significant antitumor activities [13]. Nucleases I consist of a single polypeptid chain cross-linked by two disulfide bonds [10]. Similar disulfide (S-S) linkages stabilizing structure is important feature characteristic for bovine seminal ribonuclease (BS-RNase) [13]. From this reason we decided to compare PN nuclease, in some parts of experiments, with animal ribonucleas and DNase I, which are not characteristic by disulfide linkages. The commercial Mung bean nuclease was also used for the comparison.

Materials and methods

Materials. Bovine pancreatic ribonuclease (RNase A) was purchased from MP Biochemicals, Irvine, California USA. Bovine seminal ribonuclease (BS-RNase) was prepared from the bull seminal vesicle fluid [11]. WLN-RNase (wheat leaf neutral ribonuclease) was isolated from wheat leaves [40]. Bovine Deoxyribonuclease I and bovine Deoxyribonuclease II (DNase I, DNase II) were purchased from Sigma-Aldrich Corporation, St. Louis, Missouri USA. Mung bean nuclease (PhA) was supplied by MP Biochemicals. Microtiter plates (Gamma, Ceske Budejovice, Czech Republic) were used for determination of antibodies. The SwAM-Px (swine antimouse IgG with peroxidase) produced by Sevapharma, Prague Czech Republic was used for antibody titration measured with Titertek Uniskan, Flow laboratories, Irwine, UK.

Isolation and purification of pine pollen nuclease (PN). Black pine (*Pinus nigra)* pollen was collected in the June-July 2006 from matured pine male inflorescences and stored at -20 °C. Pollen diffusate was prepared approximately 60 days post collection. To prepare an aliquote of pollen diffusate, 5 g of pollen was shaken vigorously for 3 min. using a vortex mixer in 100 ml of 0.1 M sodium acetate extraction buffer pH 4.8 containing 1mM $ZnSO_4$ and 10 % sucrose. Then the diffusate was filtered using a Buchner funnel. The filtrate was stored at -20°C until used for ultrafiltration and subsequent purification. In total, 14 l of filtrate was collected that was obtained from 700 g of pollen and used on the onset of purification experiments. The nitrocellulose membrane MWCO 10000 was used for the ultrafiltration and filtrate was discarded. Retentate was applied on HiTrap SP column (4x5 ml) that had been previously equilibrated with 50 mM acetate buffer, pH 4.8. The gradient of ionic strength was used for the elution. The fractions containing nuclease activity were collected, desalted and applied to HiTrap Heparin FF16/10 column (20 ml) (Amersham, USA). Elution was carried out using increasing concentration of sodium chloride from 0 to 1 M. Enzyme was eluted in a concentration range of 0.2 M to 0.3 M sodium chloride. The fractions with nuclease activity were applied on Sephacryl S100HR column (Amersham, USA) which was equilibrated using 50 mM phospate buffer pH 6.8. Finally purified nuclease was used for the studies.

Antiproliferative activity tested in vitro on cell culture. The cell line ML-2 (derived from human myeloid leukemia) was used for testing of PN antiproliferative activity as described previously [2].

A total of $2x10^5$ cells in 0.2 ml RPMI 1640 medium supplemented with fetal calf serum (10% v/v) were established in cell-culture plate (NUNC 96, FB type, Denmark) and cultivated in humidified atmosphere containing 5% of CO_2 for 48 hrs. Simultaneously a known concentration of nucleases, DNase I and bovine RNases was added to each triplicate culture. Four hours before the termination of cultivation, the samples were pulsed with 24 kBq of [6-^{3}H]-thymidine (specific activity 980 GBq/mmol). Cells were than collected with a Scatron cell harvester and radioactivity measured in beta counter Beckman. The mean values of the triplicates containing a particular ribonuclease were compared with that of untreated control cells and expressed in percent of cell proliferation.

In vivo treatment of PN nuclease on melanoma xenografts. The antitumoral activity of PN nuclease and comparative substances was tested on athymic female nude mice CD-1 weighing 19-21g. The mice were housed under aseptic conditions in cages with bedding (SAWI Research bedding sterilized by irradiation) and fed with sterile diet as referred previously (25). Human melanoma was obtained by means of melanoma cell line C-32 applicated subcutaneously on the right flank of the mouse. Treatment of the mice started when the area of transplanted tumor reached 5x5 mm. PN nuclease (10μg/20g) and some comparative substances were administered intratumorally (i.t.) 3 times a week in the course of a three-week period. Control animals received equal volumes of vehicle (saline) administered according to the same schedule. The effects of PN nuclease and other substances on tumor growth were quantitated by measuring tumor size in three dimensions (lenght × width × depth) with Vernier caliper [18].

Spermatogenic toxicity in mice. The aspermatogenic effect of isolated PN nuclease and comparative enzymes was determined as described previously [22]. Adult male ICR mice were injected with 10 μg of PN nuclease, PhA nuclease or 100mg of BS-RNase, RNase A, DNase I and DNase II dissolved in phosphate buffered saline ones a week during five weeks. The mice were also injected with 10 mg PN nuclease ones into left testes and by PN dissolved in immune mice anti PN nuclease serum and sacrificed after 10 days to determine the effect of immune antibodies on the biologic activity of injected PN nuclease, in comparison with the same injections without anti PN serum. The injected testes without epididymis were excised and histologically studied. Destructive effects on the testes were detected by the decrease in the width of spermatogenic layers and the

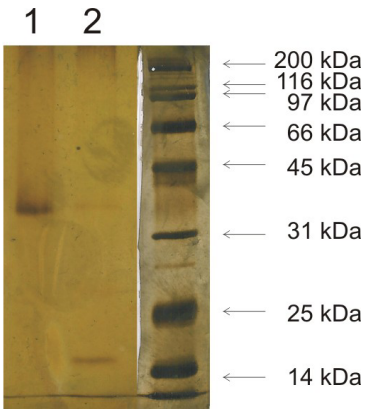

Figure 1. SDS polyacrylamide electrophoresis (12 %, Tris-glycine, pH 8,8 electrode buffer) of nuclease from black pine pollen (PN). Purified sample under non-reducing (without dithiotreitol), (Line1) and reducing (with dithiotreitol) condition (Line2).

diameter of seminiferous tubules. 50 and 60 tubules from central part of the testes were measured under microscope equipped with micrometer scale.

Embryotoxic effect. The effect of PN and PhA nucleases, DNases, RNases A and BS-RNase on the development of mouse embryos *in vitro* was assayed. Two cell embryos of superovulated mice were flushed from oviducts cca 36 hrs after mating. Embryos were cultured in CZB medium supplemented with bovine serum albumin (3 mg/ml) and with added ribonuclease at the dose of 100 mg/ml for 72-96 hrs at 37°C in a humidified atmosphere containing 5% of CO_2. The development stage of embryos was evaluated by microscopy [19].

Immunogenicity determination. The immunogenicity of all enzymes was determined as described previously [20]. A non-competitive ELISA test was performed. Microtiter plate wells were coated with 25 μg of all studied nucleases, and ribonucleases. After washing the plates, antibody from mice treated with the above mentioned enzymes and control sera from mice injected with PBS were serially diluted in wells and incubated at 37°C for 2 hrs. The Sw AM-Px (swine anti-mice IgG with peroxidase) conjugate 1:1000 was added, and after 20 min of incubation with the tetramethylbenzidine substrate solution, the reaction was stopped with addition of 4.0 N H_2SO_4. The antibody reaction was measured using photometry at 450 nm. The tests were defined as positive when optical density of the serum tested was found to be least three standard errors of the mean (S.E.M.) higher than that of control mice injected with PBS.

Complex of PN nuclease with antibodies and its in vivo studies on mice testes.

The sera from mice injected by PN nuclease exhibiting the titer of antibodies from 320 to 640 were pooled and used for antigen-antibody tests. Fifty microliters of pooled serums were mixed with 500 μg of isolated PN and tested *in vivo* by injecting of 10 μl of this mixture into left testes of five mature mice. The mice were killed after 10 days and the width of spermatogenic layers and diameter of seminiferous tubules were histologically tested.

Histology. All animals injected with PN and comparative substances were subjected to excision of testes and tumor enucleation. The small pieces of these tissues were consequently fixed in Bouin solution for histology examination. The fixed samples of tissues were embedded in paraffin blocks. Tissues slides (5 mm) were cut and stained with hematoxylin-eosin.

Statistical analysis. The results are presented as mean ± SEM. The data were analyzed statistically using Fisher´s *t*-test.

Ethics. All the mentioned experiments adhered to ethical standards and were approved by the institutional committee (approval no. 3/04) all researchers handling experimental animals possess certificates from the Central Committee for Animal Welfare.

Results

Antiproliferative effect of PN nuclease on ML-2 tumor cell line in vitro and antitumor effect in vivo after its intratumoral (i.t.) application in nude mice bearing human melanoma tumor. Purity of isolated PN nuclease (see *Isolation and purification of pine pollen nuclease (PN)*) was proved by means of gel electrophoresis (Fig. 1). The action of PN nuclease and the comparative enzymes on human ML-2 cell line demonstrated that the inhibitory effect of PN nuclease on cell proliferation has been very

Table 1. Growth of human melanoma tumors in athymic mice after intratumoral injection of *Pinus nigra* pollen (PN) in comparison with *Phaseolus aureus* nuclease (PhA) and bovine ribonucleases RNase , BS-RNase and DNase I

Substances injected (μg)	Number of		Tumor volume after injections (cm^3)	Percent loss of tumor volume compared with control %	Degree of tumor degeneration	Body mass (g) before	after
	mice	injection				injections	
PBS (Control)	5	9	2.41 ± 0.4	0	0	23 ± 1	24 ± 1
RNase A (100)	4	9	2.36 ± 0.3	0	0	22 ± 2	24± 1
BS RNase (100)	5	9	0.52 ± 0.07^{++}	79^{++}	4	24 ± 1	21± 3
DNase I (80)	5	7	1.08 ± 0.6	55^{++}	3	23 ± 2	17± 3
PhA nuclease (10)	5	9	0.83 ± 0.04^{++}	65^{++}	4	23 ± 1	21± 2
PN nuclease (10)	4	9	1.16 ± 0.29	46^{++}	3	24 ± 2	24 ± 1

$^{++}$ $P < 0.01$

Degree of tumor degeneration> 0 – without degeneration, 3 – 40 – 60 and 4 – 60 – 90 % degeneration

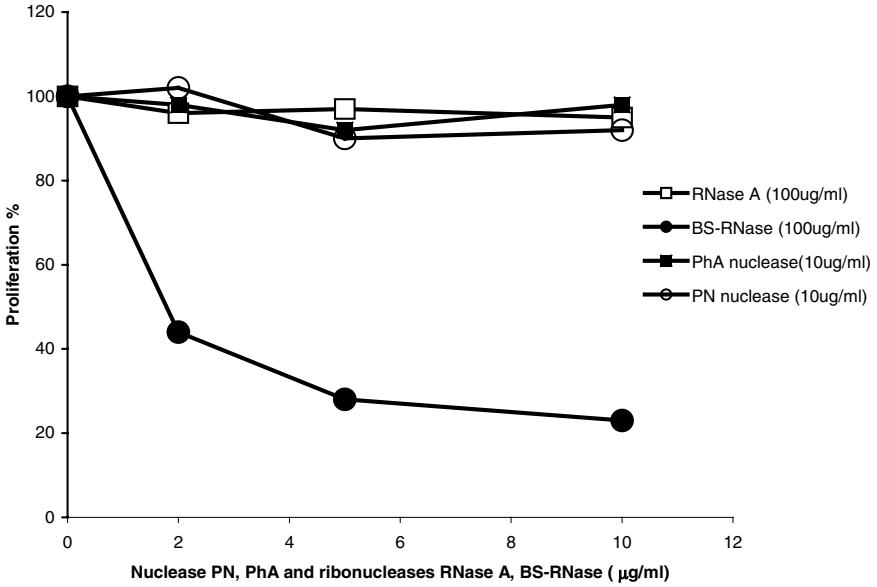

Figure 2. Proliferation of human ML-2 cells incubated with PN nuclease and comparative enzymes PhA nuclease,RNase A and BS-RNase.

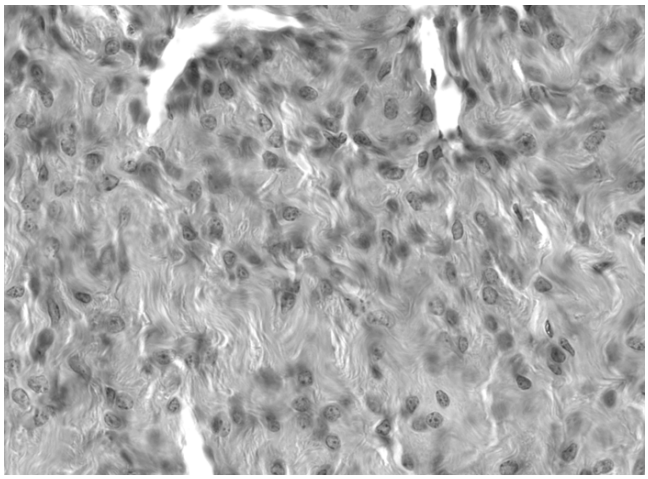

Fig. 3a. Effect of PN nuclease injected 6 times intratumorally into athymic mice bearing human melanoma tumors.Tumor cells and gradually the whole tissues was ligamentously degenerated by polymorphonuclears,monocysts,fibroblasts and fibrocysts. Together with this degeneration the tumor size also decreased (see Table 1)

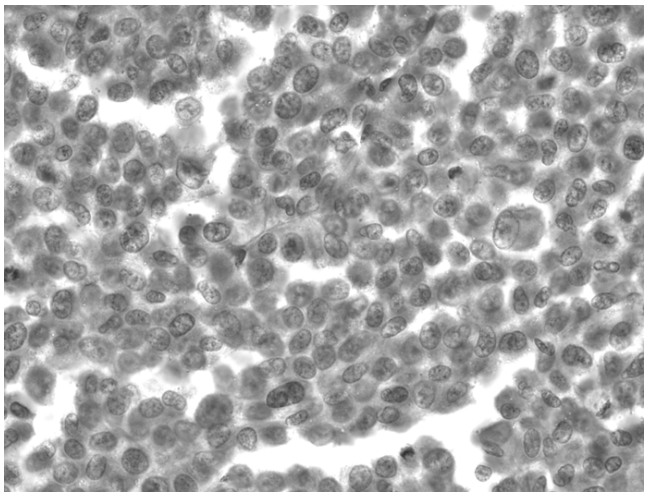

Fig.3b.Effect of bovine pancreatic RNase A injected 6 times intratumorally into athymic mice bearing human melanoma tumors. Tumor cells and tissue do not shown degeneration and the size of tumors did not decrease.

weak, similarly as that of comercial plant PhA nuclease and RNase A ribonuclease, meanwhile, BS-RNase, antiproliferative activity appeared to be markedly expressed (Fig. 2).

On the other side PN nuclease (10 mg per injection) after seven applications into melanoma tumors growing in nude mice exerted reduction and degradation of these tumors, without toxic effect on the body weight of mice (Table 1, and Fig.3a). Similar effect was observed also with commercial PhA nuclease (10 mg i.t.), DNase I and BS-RNase (80 and 100mg i.t.) after their application into melanoma tumors growing in nude mice.

Effect of PN nuclease and comparative enzymes on mice spermatogenesis and on development of mice early embryos as a certain manifestation of side effects. The aspermatogenic effects of PN nuclease and comparative enzymes, obtained after intratesticular and intraperitoneal application to mice, are given in Table 2 and 3. The application of the PN nuclease into testes by 10μg depressed significantly only width of spermatogenic layers, the injections of BS-RNase (Table 2) depressed all spermatogenic parameters. After the i.p. injections of PN nuclease the spermatogenic depression was

Table 2. Aspermatogenic effect of PN nuclease injected into left testes in comparison with PhA nuclease and bovine ribonucleases (RNase A, BS-RNase)

Substances injected (μg)	No. of mice	Index weight of testes ± SEM		Width of spermatogenic layers ± SEM		Diameter of seminiferous tubuli ± SEM	
		Injected testes	Non-injected testes	Injected testes	Non- injected testes	Injected testes	Non-injected testes
PBS	5	44 ± 7	47 ± 3	61 ± 5	63 ± 3	161 ± 5	163 ± 4
RNase A (100)	5	42 ± 6	43 ± 6	62 ± 5	61 ± 6	164 ± 4	166 ± 5
BS-RNase (100)	5	39 ± 4	39 ± 5	24 ± 6^{++}	59 ± 8	142 ± 10^{++}	150 ± 17
Ph A nuclease (10)	5	22 ± 4^{++}	44 ± 3	5 ± 9^{++}	49 ± 7^{++}	98 ± 22^{++}	143 ± 5
PN nuclease (10)	12	53 ± 6	48 ± 4	34 ± 2^{++}	53 ± 5	152 ± 9	154 ± 5

++ $P < 0.01$

Table 3. Aspermatogenesis and antigenicity (antibody titres) of PN nuclease after i.p. injections in comparison with PhA nuclease and bovine ribonucleases RNase A and BS-RNase

Substances injected (μg)	No. of mice	Index weight of both testes ± SEM	Width of spermatogenic layers of both testes in μm ± SEM	Diameter of seminiferous tubules of both testes in μm ± SEM	Titre of antibodies against homologous substances
PBS (control)	5	98 ± 7	65 ± 6	165 ± 7	0
RNase A (100)	5	95 ± 6	64 ± 5	166 ± 5	80 – 160
BS RNase (100)	5	81 ± 9^{++}	45 ± 6^{++}	161 ± 7	1280 – 2560
PhA nuclease (10)	5	88 ± 4	43 ± 2^{++}	159 ± 4	320 – 640
PN nuclease (10)	3	90 ± 7	42 ± 3^{++}	160 ± 5	160 – 320

$^{++}$ $P < 0.01$

Table 4. Development of mice embryos after 72 hours incubation with PN nuclease compared with PhA nuclease (10μg of nucleases in 1 ml of medium)

Enzymes species Added to medium used for embryos culture	No. of mice embryos	Number of embryos in cell stages				Embryous splitting	
		Explanded blastocysts	Blastocysts	4 – 8 cell embryos	No. of degenerated embryos	No.	%
Control	17	8	3	1	5	12	70
PhA	20	0	2	7	11	9	41^{+}
PN	16	0	0	5	11	5	31^{++}

$^{+}$ $P < 0.05$, $^{++}P < 0.01$

found only in the width of spermatogenic layers, meanwhile BS-RNase was toxical also in the index weight (Table 3). Embryotoxical effect of PN nuclease was equal to that of commercial PhA nuclease (Table 4). At all events the embryotoxic activity of PN nuclease was not so strong as that of onconase (ribonucleotic enzyme isolated from frog eggs and early embryos) [20].

Complex of PN nuclease with its antibodies and its activity on mice spermatogenesis. In the former study [22] there was found that antibodies against BS-RNase block out the cytotoxic effect of this ribonuclease *in vitro*. In these experiments, we tested the effect of PN nuclease injected with its mice homologous antiserum *in vivo.* When the PN nuclease in complex with mice antiserum (mice five times injected by 10mg of PN nuclease and their antisera were joined) was injected to the left testes of mice, the marked drop of spermatogenesis appeared in the higher level as with free PN nuclease. Width of spermatogenic layers of testes injected by free PN was determined as 42 ± 3 μm while PN with antiserum dropped significantly to 28 ± 8 μm which result is significantly less ($P < 0.01$). Diameter of spermatogenic tubules moved above the 150μm. This result confirmed that anti-PN antibodies are not able to inactivate the PN nuclease injected.

Immunogenicity and antigen relationship between PN nuclease and other enzymes. The immunogenicity expressed as production of antibodies in the mice injected i.p. with plant PN and PhA nuclease and animal RNase A appeared to be low-titres 10 – 640 (Table 3). An exception was the production of antibodies against animal BS-RNase in which titers ranged from 1280 to 2560. The titer of these antibodies is significantly higher in comparison with PN nuclease. All antibodies against animal enzymes reacted with PN nuclease in the titer 10 – 40 included plant WLN-RNase (wheat leaf neutral ribonuclease) studied in last year (40). An exception is

PhA nuclease in which its antibodies reacted with PN nuclease antigens in the titer 320-640. The same titer 320-640 was noticed with the homologous antibodies against PN nuclease. The mutual immunological reactivity (cross-reaction) of PN nuclease with other plant and animal studied enzymes seems to prove that the black pine pollen nuclease (PN) resembles structurally (antigenically) not only to commercial mung bean sprout nuclease (PhA) and WLN-RNase but also to animal enzymes and vice versa (data not shown).

Discussion

Plant nucleases I, major plant sugar nonspecific endonucleases, forms rather heterogenous group of catalytically related enzymes. Their activity has been reported in a number of plant species like pollen extracellular nuclease from evolutionary old *Pinus nigra* [23] and through many other species of plants [2, 24-36]. All these species demonstrate clearly that sugar nonspecific nuclease I is an universal enzyme, widely spread in higher plants.

Important properties that suggest intimate heterogeneity within the nuclease I group are the specificity towards secondary structures of nucleic acids and its ability to cleave different homopolymers. There are on the one hand enzymes strongly single strand specific and on the other hand enzymes having ability to cleave different secondary structures, including double-stranded DNA or supercoiled plasmid DNA as well as dsRNA [2, 4 – 12, 37].According to very limited literature data available so far, an antiproliferative activity from roots or mature seeds were isolated [34-36]. All of the substances of mentioned plant parts were studied of course under *in vitro* condition. While some plant nucleases have similar molecular mass as animal ribonucleases [13-15] and nuclease I consists of a single polypeptide chain cross linked by two disulfide bonds as bovine seminal BS-RNase [13], we decided to compare black pine nuclease (PN) with commercial mung been nuclease (PhA), and partly with WLN-RNase [40] and bovine DNases and RNases.

The immunogenicity of PN nuclease, which is also characterized by a certain intensity of antibody production, was similar to that of PhA, and RNase A. However, BS-RNase has most immunogenicity in comparison with foregoing enzymes. This observation is surely positive for PN nuclease administration, since the production of antibodies would not be so intensive as the production of antibodies after BS-RNase injections. The complex of PN nuclease with homologous antibodies and injected into left testes of mice degenerated spermatogenic epithelium by the higher intensity as PN nuclease alone, without antibodies. This situation proves that antibodies against PN nuclease did not block the aspermatogenic effect of this nuclease and probably the antiproliferative activity. It is possible to suppose that this phenomenon could be the same or similar to other biological activities in mice injected by BS-RNase, PhA and WLN-RNase complex with antibodies or with BS-RNase, PhA nuclease and WLN-RNase alone (without antibodies)[2, 20, 39]. Of course it would be also possible that these complexes are not stable and they are disconnected in the *in vivo* setting.

Spermatogenic and early embryonic cells are very effective markers for studying the toxic and side effects of various substances injected into animals. This is the reason, why mice testes and early embryos were used for testing the toxicity of PN nuclease. Less toxicity of PN nuclease in comparison with BS-RNase (results in these experiments) and onconase [20] are promising for the further studies of this and other plant nucleases for their eventual lower cytotoxicity.

Regarding the experiments *in vitro* on human tumor cell line ML-2 the PN and also PhA nucleases did not show any remarkable antiproliferative effect. On the other side, the results of their *in vivo* experiments on human melanoma tumors growing in athymic mice were promissing. PN even PhA [2] nucleases intratumorally injected performed almost ten times higher antitumor effects in comparison with BS RNase and WLN-RNase. Similarly to the results with WLN-RNase [40] the antitumor activity of PN was preliminary proved also by intravenous injections of this enzyme conjugated with PEG (results are not shown).These experiments confirmed that 5μg of PN nuclease exerted a similar antitumor activity as 50μg of BS-RNase and WLN-RNase conjugated to PEG [40].

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References

[1] AOYAGI M, SUGIYAMA M, FUKUDA H. BENI and ZENI cDNAs enconding SI-type DNase that are associated with programmed cell death in plants. FEBS Lett 1998; 429: 134–138.

[2] SOUČEK J, ŠKVOR J, POUČKOVÁ P, et al. Mung bean (*Phaseolus aureus*) sprout nuclease and its biological and antitumor effects. Neoplasma 2006; 53: 402–409.

[3] GITE SU, SHANKAR V. Single-strand-specific nucleases. Crit Reb Microbiol 1995; 21: 101–122.

[4] WILSON CM. Plant nuclease: biochemistry and development of multiple molecular forms. Isozymes Curr Top Biol Med Res 1982; 6: 33–54.

[5] DANGL JL, DIETRICH RA, THOMAS H. Senescence and programmed cell death. In: Buchman B, Gruissen W, Jones R, editors. Biochemistry and Molecular Biology of Plants, Rockville, M.D,.2000: 1044–1100.

[6] ITO JH, FUKUDA J. Is a key enzyme in the degradation of nuclear DNA during programmed cell death of tracheary elements. Plant Cell 2002; 14: 3201–3211.

[7] PÉREZ-AMADO MA, ABLER ML, DEROCHER J, THOMPSON DM, VAN HOOF A, et al. Identification of BFNI, a bifunctional nuclease induced during leaf and stem senescence in Arabidopsis thaliana. Plant Physiol 2000; 122: 169–1

[8] WOOD M, POWER JB, DAVEY MR, et al. Factor affecting single strand-preferring nuclease activity during leaf aging and dark-induced senescence in barley (*Hordeum vulgare L.*), Plant Sci 1998; 131: 149–159.

[9] ZAINAA C, MARASSUTTIA C, de AMICISA F, et al. Endonuclease genes up-regulated in tissues undergoing programmed cell death are expressed during male gametogenesis in barley. Gene 2003; 315: 43–50.

[10] WILSON, CM. Plant nuclease. Ann Rev Plant Physiol 26: 187–208.

[11] KOWALSKI D, KROEKER WD, LASKOWSKI M. Mung bean nuclease I. Physical, chemical, and catalytic properties. Biochemistry 1976; 15: 4457–4463.

[12] NASEEM I, HADI SM. Single-strand-specific nuclease of pea seeds: Glycoprotein nature and associated nucleotidase activity. Arch Biochem Biophys 1987; 255: 437–445.

[13] D´ALESSIO G, RIORDAN JF, editors. Ribonucleases: Structures and Functions. San Diego,Academic Press, 1997.

[14] MATOUŠEK J. Ribonucleases and their antitumor activity. (Review) Comp Biochem Physiol C 2001; 129: 175–191.

[15] MAKAROV AA, ILINSKAJA ON. Cytotoxic ribonucleases: molecular weapons and their targets. FEBS Lett 2003; 540: 15–20.

[16] SOUČEK J, MARINOV I, BENEŠ J, et al. Immunosuppressive activity of bovine seminal ribonuclease and its mode of action. Immunobiology 1996; 195: 271–285.

[17] LELAND PA, SCHULTZ LW, KIM B, et al. Ribonuclease A variants with potent cytotoxic activity. Proc Natl Sci USA, 1998: 10402–10412.

[18] POUČKOVÁ P, ZADINOVÁ M, HLOUŠKOVÁ D et al. Polymer- conjugated bovine pancreatic and seminal ribonucleases inhibit growth of human tumors in nude mice. J Control Release 2004; 95: 83–92.

[19] MATOUŠEK , GOTTE G, POUČKOVÁ P, et al. Antitumor activity and other biologic actions of oligomers of ribonuclease A. J Biol Chem 2004; 278: 23817–23822.

[20] MATOUŠEK J, SOUČEK J, SLAVÍK T, et al. Comprehensive comparison of the cytotoxic activities of onconase and bovine seminal ribonuclease. Comp Biochem Physiol C. 2003; 136: 343–356.

[21] MATOUŠEK J, POUČKOVÁ P, SOUČEK J, et al. PEG chain increase aspermatogenic and antitumor activity of RNase A and BS RNase enzymes. J Control Release 2002; 82: 29–37.

[22] MATOUŠEK J. Aspermatogenic effect of the bull seminal ribonuclease (BS-RNase) in the presence of anti BS-RNase antibodies in mice. Anim Genet 1994; (Suppl 1) 25: 45–50.

[23] MATOUŠEK J, TUPÝ J. The release and some properties of nuclease from various pollen species. J Plant Physiol 1985; 119: 169–178.

[24] MATOUŠEK J, TRNĚNÁ L, OBERHAUSER R, et al. dsRNA degrading nucleases are differentially expressed in tobaco anthers. Biol Chem Hoppe Seyler 1994; 375: 261–269.

[25] MATOUŠEK J, TUPÝ J. Purification and properties of nuclease from tobacco pollen. Biol Plant 1984; 26: 62–73.

[26] MATOUŠEK J, TURKOVÁ V. Some immunochemical properties of pollen extracellular nuclease. Biol Plant 1987; 29: 401–408.

[27] OLSEN AE, JANSKI A, CLARK ET. An extracellular nuclease from suspension cultures of tobacco. Biochem Biophys Acta 1974; 336: 89–100.

[28] THOM M, MARETZKI A, KOMOR E. Vacuoles from sugarcane suspension cultures. Plant Physiol 1982; 69: 1315–1319.

[29] ABEL S, GLUND K. Localization of RNA-degrading enzyme activity within vacuoles of cultured tomato cells. Physiol Plant 1986; 66: 79–86.

[30] THELEN MP, NORTHCOTE DH. Identification and purification of a nuclease from Zinnia elegans L.:a potential molecular marker for xylogenesis. Planta 1989; 179: 181–195.

[31] MATOUŠEK J, TRNĚNÁ L, SVOBODA P, et al. The gradual disappearence of hop latent viroid (HLVd) from hop mericlones following heat therapy: a possible role for a nuclease degrading dsRNA. Biol Chem Hope Seyler 1995; 376: 715–721.

[32] YUPSANIS T, ELEFTHERIOU P, KELEPIRI Z. Separation and purification of both acid and neutral nucleases from germinated alfaalfa seeds. J Plant Physiol 1996; 149: 641–649.

[33] BLANK A, MC. KEON TA. Single-strand-preferring nuclease activity in wheat leaves is increased in senescence and is negatively photo regulated. PNAS USA 1989; 86: 3169–3173.

[34] LAM SK, NG TB. Isolation of a novel thermolabile heterodimeric ribonuclease with antifungal and antiproliferative activities from roots of the Sanchi ginseng panax notoginseng. Biochem Biophys Res Commun 2001; 285: 419–423.

[35] XIA HC, FENG LI, ZHEN LI, et al. Purification and Characterization of Moschatin, a novel type I ribosome-inactivating protein from the mature seeds of pumpkin (*Cucurbita moschata*) and preparation of its immunotoxin against human melanoma cells. Cell Res 2003; 13: 369–374.

[36] XU H, W-XA LIU WJ. A novel ribotoxin with ribonuclease activity that specifically cleaves a single phosphodiester bond in rat 28S ribosomal RNA and inactivates ribosome. Arch Biochem Biophys 2004; 427: 30–40.

[37] CHANG SC, GALLIE DR. RNase activity decreases following a heat shock in wheat leaves and correlates with its post translation modification. Plant Physiol 1997;113: 1253–1263.

[38] GREEN PJ. The ribonucleases of higher plants. Ann Rev Plant Physiol Plant Mol Biol 1994; 45: 421–445.

[39] BUFE A, UHLIG U, SHOLZEN T, et al. A nonspecific singlestranded nuclease activity with characteristics of a topoisomerase, found in a major grass pollen allergen: Possible biologic significance. J Biol. Chem 1999; 380: 1009–1010.

[40] ŠKVOR J, LIPOVOVÁ P, POUČKOVÁ P, et al. Effect of wheat leaf ribonuclease on tumor cells and tissues. Anti-Cancer Drugs 2006; 17: 815–823.