Characterization of *Aspergillus niger* B-1 RNase and Its Inhibitory Effect on Pollen Germination and Pollen Tube Growth in Selected Tree Fruit

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ABSTRACT. *Aspergillus niger* B-1 (CMI CC 324626) extracellular RNase (RNase B1) was purified to homogeneity. It was found to contain two isoforms of 32- and 40-kDa glycoproteins, sharing a 29-kDa protein moiety. Optimal RNase activity was observed at 60 °C and pH 3.5. In 'Almog' peach [*Prunus persica* (L.) Batsch (Peach Group) 'Almog'] and 'Murcott' tangerine (*Citrus reticulata* Blanco 'Murcott') the enzyme inhibited pollen germination and pollen tube growth in vitro as well as in vivo. In field experiments, spray application of the RNase caused a reduction in 'Fantasia' nectarine [*Prunus persica* (L.) Batsch (Nectarine Group) 'Fantasia'] fruit set and interfered with embryo development. The biological effect of the RNase may be of horticultural value, due to its potential to control fertilization.

In self-incompatible plants, growth of self-pollen is arrested in the stigma or style, before fertilization occurs. Self-incompatibility is often controlled by a single multiallelic *S*-gene (de Nettancourt, 1977). A common outbreeding mechanism is gametophytic self-incompatibility, in which a pollen tube bearing an *S*-allele identical to one of the two alleles of the pistil fails to achieve fertilization (Clarke and Newbigin, 1993; Haring et al., 1990; Hinata et al., 1993; Newbigin et al., 1994; Singh and Kao, 1992). In members of Solanaceae and Rosaceae, the *S*-alleles have been found to encode stilar-specific *S*-glycoproteins exhibiting ribonuclease (RNase) activity, as well as the ability to inhibit the growth of self-pollen tubes (Clarke et al., 1990; Kaufmann et al., 1991; Kirch et al., 1989; McClure et al., 1989; Sassa et al., 1992). Two domains in the primary structure of these *S*-glycoproteins, containing one histidine residue each show strong homology to *Aspergillus oryzae* (Ahlburg) Cohn RNase T₂, *Rizopus niveus* Yamazaki IFO 4810 RNase Rh (McClure et al., 1989), and *A. saitoi* Sakaguchi et al. RNase M (Watanabe et al., 1990).

In some plants non-*S*-allele-specific ribonucleases were found to be produced by the female organs. For example, RNase X has been reported to be shared by both self-incompatible *Nicotiana glauca* Link et Otto and self-compatible *N. tabacum* (L.) (McClure et al., 1989). In self-incompatible *Petunia inflata* Fries RNase X₂ shares homology with *S*-RNases, yet its gene is not located on the *S*-locus (Lee et al., 1992). The biological traits and function of these RNases were not fully determined yet. In self-compatible peach [*Prunus persica* (Peach Group)] and calamondin (*Citrus reticulata* 'Austera' × *Fortunella* sp. Swingle) the stigmatic diffusate contains RNase activity (Roiz and Shoseyov, 1995; Roiz et al., 1995). These stigmatic RNases inhibit pollen germination and tube elongation in vitro in a dose-responsive manner. Furthermore, it was shown in the above studies that bovine pancreatic RNase A and *A. oryzae* RNase T₁, which are nonspecific, had similar inhibitory effect in peach and calamondin, respectively. In both species in vitro studies suggest that non-*S*-allele RNases play a role in the pollination process.

The effect of nonspecific RNase may be of horticultural value.

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In many deciduous fruit trees, yields of commercial value depend upon massive thinning of the flowers and fruitlets. In stone fruits [peach, plum (*Prunus domestica* L.), etc.] the thinning process is currently performed manually. In *Citrus* L. trees, on the other hand, seedless fruits are desirable. Therefore, a further step in this research was to examine an effective RNase that can be produced in large amounts. Species of *Aspergillus* have been reported to produce extracellular RNases (Egami and Nakamura, 1969). *Aspergillus niger* is also used as a source for important materials and enzymes in the food industry, such as citric acid and pectinases (Kerns et al., 1987; Li and King, 1963; Rombout and Pilnik, 1978). In the present work we describe production, purification, and partial characterization of *A. niger* B-1 extracellular RNase (RNase B1) and its effect on in vitro and in vivo pollen germination and pollen tube growth.

Materials and Methods

PREPARATION AND PURIFICATION OF *A. niger* EXTRACELLULAR RNASE. *Aspergillus niger* B1 (CMI CC 324626) was grown in liquid culture containing 1% (w/v) wheat flour and 0.05% (w/v) (NH₄)₂SO₄. The mixture was adjusted to pH 3.5 with HCl and autoclaved. An inoculum of ≈10⁶ spores was suspended in 100 mL of medium and incubated at 30 °C in an orbital shaker, at 200 rpm for 100 h. The growth medium was passed through a 0.2-μm membrane and dialyzed three times against 10 volumes of 2 mM sodium acetate pH 6. Two liters of dialyzed solution were loaded onto a Fractogel EMD-TMAE 650 (M) 26/10 (Merck, Darmstadt, Germany) column, equilibrated with 20 mM sodium acetate pH 6. Bound proteins were eluted with a 500-mL linear gradient of 0 to 1.0 M NaCl in the same buffer, using a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) at a flow rate of 5 mL·min⁻¹. The fractions exhibiting the highest RNase activity were pooled and dialyzed against 2 mM sodium acetate pH 6, and a 50-mL aliquot was loaded onto a Mono Q 5/5 HR (Amersham Pharmacia Biotech) column, equilibrated with 20 mM sodium acetate pH 6. The elution was performed as with the EMD-TMAE column, except that only 10 mL of a 0 to 1.0 M salt gradient were used, at a flow rate of 1 mL·min⁻¹.

Proteins were monitored at 280 nm and measured according to

Bradford (1976), using bovine serum albumin (BSA) as a standard. Different fractions were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970). An RNase activity gel was run according to Roiz and Shoseyov (1995).

The purified RNase B1 was deglycosylated enzymatically according to the procedure of Broothaerts et al. (1991). The enzyme was mixed with 0.5% (w/v) SDS and 5% (w/v) β -mercapthoethanol and heated at 100 °C for 5 min. Once cooled, the reaction mixture was diluted 2.5-fold with buffer containing 50 mM sodium phosphate pH 7.5, 25 mM EDTA, 1% (w/v) Triton X-100, and 0.02% (w/v) sodium azide. Peptide-N-glycosidase F (PNGase F, Boehringer-Mannheim GmbH, Mannheim, Germany) was added to a final concentration of 20 units/mL and incubated overnight at 37 °C. The sample was then mixed with sample application buffer, heated at 100 °C for 5 min, and analyzed by 12.5% SDS-PAGE.

RNase ASSAYS. The optimal conditions for RNase activity were determined within a range of temperatures from 20 to 100 °C at 10 °C intervals, and across a pH range from 2.5 to 7 at 0.5 pH units intervals, using phosphate-citrate buffer (50 and 12 mM, respectively). The RNase activity assay was modified from Brown and Ho (1986). Samples of 10 μ L were added each to 490 μ L of ice-cold buffer, containing 4 mg·mL⁻¹ yeast RNA (Sigma, St. Louis, Mo). Half of the mixture was moved immediately to another tube containing 50 μ L 0.75% (w/v) uranyl sulfate in 25% (w/v) perchloric acid (stop mixture), for use as a blank. The rest was incubated for 10 min, then 50 μ L of stop mixture were added. Following centrifugation at 15,000 g_n for 5 min, the supernatant was diluted 20-fold with distilled water and absorbance was determined at 260 nm. One unit of RNase activity was determined as the amount of enzyme releasing soluble nucleotides at a rate of one A 260 nm·min⁻¹.

RNase B1 was visualized by an activity gel, as modified from Roiz and Shoseyov (1995). An SDS gel containing RNase B1 was renatured by washing twice for 15 min each with 20 mM sodium acetate pH 3.5 containing 25% (v/v) isopropanol and then twice for 15 min each with buffer alone. The renatured gel was laid over a plate containing 0.1% RNA and 0.8% agarose in 20 mM sodium acetate and incubated at 37 °C for 30 min. The gel was then removed and the agarose plate was stained with 0.02% (w/v) toluidine blue in water to visualize RNase activity.

EFFECT OF RNase B1 ON POLLEN TUBE GROWTH. Pollen of 'Almog' Peach germinated in vitro in liquid culture, as described by Roiz and Shoseyov (1995). Pollen grains were suspended in aliquots containing 100 μ L 15% (w/v) sucrose, 100 μ g·mL⁻¹ boric acid, 200 μ g·mL⁻¹ magnesium sulfate, 200 μ g·mL⁻¹ calcium nitrate and different concentrations of RNase B1. After incubation overnight at 25 °C in a dark chamber, percent germination was recorded. Pollen tube length was examined by light microscopy with an eyepiece micrometer.

The effect of RNase B1 treatment on pollen tube growth was tested also in vivo. In peach and in tangerine (*Citrus reticulata* 'Murcott') intact flowers at the very beginning of anthesis, were sprayed with RNase B1 diluted in 20 mM citrate buffer pH 3.5 to a concentration of 0.2 mg·mL⁻¹ protein, having 100 units/mL RNase activity. In each species additional flowers at the same stage, on different branches, were sprayed with buffer alone or remained nontreated as controls. After exposure to open pollination for 48 h, the styles were fixed in 3 acetic acid : 1 ethanol (by volume) for 24 h, washed with distilled water and imbibed overnight in 8 M NaOH. Following thorough washes in distilled

water, the styles were cut longitudinally, immersed each in a drop of 0.1% (w/v) aniline blue in 0.1 M potassium phosphate on a slide and carefully squashed with a cover slip. Pollen tubes were observed by epifluorescence microscopy (model BX40, equipped with WIB cube; Olympus Optical Co., Hamburg, Germany).

THE EFFECT OF RNase B1 ON FRUIT SET. Field experiments were conducted in April 1994 using trees of 'Fantasia' nectarine [*Prunus persica* (Nectarine Group) 'Fantasia'] growing in Rosh Zurim, Israel. Branches 30 to 40 cm long, bearing $\approx$ 10% open flowers, were sprayed with different concentrations of RNase B1 in 20 mM citrate buffer pH 3.5 and 0.025% triton-X 100. Nontreated branches, and branches sprayed with only buffer and triton-X 100, served as controls. The branches were sprayed at 2- to 3-d intervals during the blooming period (14 d). A month later, the number of fruit per branch was examined. For viability tests, seeds were cut longitudinally through the embryo and immersed in one percent 2,3,5-triphenyl tetrazolium chloride in water for 4 h at 20 °C in the dark. Red staining indicated viable tissues.

Results

PURIFICATION AND CHARACTERIZATION OF RNase B1. *Aspergillus niger* grown in liquid culture produced considerable amounts of extracellular RNase. A temperature of 60 °C and pH 3.5 were found optimal for RNase activity (Fig. 1), and were adopted as the standard conditions for RNase assays.

Purification of RNase B1 consisted of two steps (Table 1). The crude filtrate contained 1000 units/mL and 0.05 mg·mL⁻¹ protein. The pooled fractions around the active RNase peak eluting at 0.62 M NaCl (Fig. 2A) from an EMD-TMAE column, contained 0.1 mg·mL⁻¹ protein, with an RNase activity of 40,000 units/mL. In the final step, the RNase was eluted from a Mono Q column at 0.5

Fig. 1. Activity plots of RNase B1 as a function of (A) temperature and (B) pH.

Table 1. Purification of RNase B1 from growth medium filtrate of *A. niger* B-1. Protein concentration and RNase activity were monitored during enzyme purification.

Purification step	Total vol of extract (mL)	Total RNase activity (units $\times 10^3$)	Protein concn (mg·mL ⁻¹)	Recovery (%)	Specific activity units $\times 10^3$ /mg protein)
Crude filtrate	2000	2000	0.05	100	20
EMD-TMAE column	28	1120	0.1	56	400
Mono-Q column	1.2	652	1.05	32.6	517

Fig. 2. Anion exchange chromatography of RNase B1. The solid line = absorbance at 280 nm and the dashed line = RNase activity. (A) The crude filtrate was dialyzed and loaded onto an EMD-TMAE column. Each 7-mL fraction was located in a separate tube. (B) Fractions eluted in 0.55 to 0.65 M NaCl (A), showing highest RNase activity, were pooled and loaded onto a Mono-Q column. Each 0.5-mL fraction was collected in a separate tube.

M NaCl (Fig. 2B); the eluant had protein concentration and RNase activity of 1.05 mg·mL⁻¹ and 543,000 units/mL, respectively. Two major protein bands, of 40 and 32 kDa, were observed following SDS-PAGE of the purified RNase B1 (Fig. 3). An RNase activity gel showed active bands corresponding to the 32 and the 40 kDa proteins. When subjected to PNGase F, a single protein band appeared at 29 kDa. RNase activity was retained after PNGase digestion (data not presented).

EFFECT OF RNASE B1 ON POLLEN TUBES AND FRUIT SET. Seventy five percent of the control peach pollen grains germinated in vitro and the pollen tubes reached 0.48 ± 0.03 mm in length. Addition of RNase B1 to the growth medium reduced percentage germination and the length of the pollen tubes, in a dose responsive manner (Fig. 4). RNase B1 had a pronounced inhibitory effect, considering that 50 units/mL, representing 0.1 mg·mL⁻¹ (2.8×10^{-3} mM) protein, were found lethal, whereas BSA at a 680-fold higher concentration of 125 mg·mL⁻¹ (1.9 mM) protein, reduced only 50% of pollen germinability and tube growth.

Control pollen tubes of peach were observed growing in vivo

through the stigmatic tissue directed into the style, 48 h after pollination (Fig. 5A). A similar picture was observed in styles treated with buffer only. In contrast, pollen grains germinated on stigmas treated with RNase B1 produced short pollen tubes, which appeared to lack any growth orientation, and failed to penetrate the stylar tissue (Fig. 5B). In tangerine only a small portion of the stigmatic tissue, of which the diameter was 2 to 3 mm, could be observed in the microscopic field. Therefore, only a few pollen tubes were captured in the microscopic field, as shown in Fig. 6. However, the difference between the normal growth of the control pollen tubes (Fig. 6A) and the irregular growth of the RNase-treated pollen tubes (Fig. 6B), was observed clearly.

In 'Fantasia' nectarine RNase B1 caused a reduction in fruit set (Table 2). In nontreated branches or branches sprayed with buffer containing triton X-100, fruit set was 48.3% and 36.3%, respectively. It seemed that the low pH-buffer had some inhibitory effect on fruit set, however branches treated with 500 and 1000 units/mL of RNase B1 set 23.3% and 18.4% fruit, respectively, indicating a significant thinning effect of the RNase, with a dose dependent response.

In RNase B1 treated branches, many undeveloped fruitlets were observed. Viability tests showed that in nontreated flowers or flowers sprayed with buffer, most embryos cut surfaces were stained red, whereas embryos born in RNase-treated flowers, unevenly stained cut surfaces with brown necrotic tissues were found.

Discussion

An effective fermentation and purification process was developed for production of RNase B1 (Ozeri, 1995). A temperature of 60 °C and a pH of 3.5, values which were optimal for RNase B1

Fig. 3. SDS-PAGE zymogram of the RNase B1 purification steps. Lane 1 = crude filtrate; lane 2 = proteins eluted from the EMD-TMAE column at 0.62 M NaCl; lane 3 = proteins eluted from the Mono-Q column at 0.5 M NaCl; lane 4 = same as lane 3, except the proteins were assayed for RNase activity in situ and stained with toluidine blue; lane 5 = purified RNase after deglycosylation by PNGase; lanes 1 to 3 and 5 were stained with coomassie blue.

Fig. 4. Effect of different concentrations of *A. niger* B-1 RNase on peach pollen germination and pollen tube growth in vitro. For percentage germination, at least 100 pollen grains in each sample were monitored. For pollen tube growth, 10 pollen tubes in each sample were measured. Each point is the mean of three replications. Vertical bars = SE.

Fig. 5. Effect of RNase B1 on peach pollen tube growth in the stigma and the upper part of the style. (A) Control flower that was exposed to open pollination for 48 h. (B) Flower that was treated with RNase B1 before pollination. Scale bars = 0.2 mm.

activity, are also optimal for the activity of β -glucosidase produced by this strain (Shoseyov et al., 1988). In related species, extracellular RNases have been found to require similar conditions for optimal activity. For example, Horitzu et al. (1974) reported on *A. niger* NCR-A-1-233 RNase (50 °C, pH 3.5) and RNase T₂ (pH 4.5) and Irie (1967) reported on *A. saitoi* RNase M (50 °C, pH 4) and *Rhizopus niveus* RNase Rh (50 °C, pH 5). All these RNases have molecular weights between 24 and 34 kDa. They are also base-nonspecific enzymes with a preference to adenilic acid (Ohgi et al., 1991; Watanabe et al, 1990).

Secretion of considerable amounts of RNase should have a substantial biological role. In microorganisms, extracellular RNases are accepted generally to contribute to digestion of polyribonucleotides present in the growth medium, thereby giving rise to diffusible nutrients (Egami and Nakamura, 1969). These so-called nutritive characteristics of RNases have also been described in plants: cells of tomato (*Lycopersicon esculentum* Mill.) suspension cultures secreted RNase into the growth medium in response to phosphate starvation (Nurnberger et al., 1990). Nevertheless, more research is needed for a complete understanding of the physiological role of fungal extracellular RNases.

RNase B₁ was purified to homogeneity. PNGase deglycosylation, followed by appearance of a single 29-kDa band, indi-

Fig. 6. Effect of RNase B1 on tangerine pollen tube growth in the stigma. (A) Control flower that was exposed to open pollination for 48 h. (B) Flower that was treated with RNase before pollination. Scale bars = 0.1 mm.

Table 2. Fruit set of 'Fantasia' nectarine in Rosh Zurim, Israel. The buffer was sodium citrate, pH 3.5, containing 0.025% triton X-100. Fruit set is expressed as the percentage of developed fruit per flower.

Treatment	Flowers (total no.)	Fruit set (%)
Control nontreated	169 ^z	48.3 a ^y
Control buffer	143	36.3 b
500 units/mL RNase B1	148	23.3 bc
1000 units/mL RNase B1	106	18.4 c

^zEach value is the mean of 10 replications.

^yMean separation by Tukey-Kramer multiple range test, $P \leq 0.05$.

cates heteroglycosylation of this protein. RNase T₂ (Kanaya and Uchida, 1981) and RNase M (Ohgi et al., 1983; Watanabe 1990) have also been found to contain different isoforms, sharing the same protein moiety, but differing in carbohydrate content. In RNase T₂ the occurrence of different-size glycan chains has been suggested to be due to enzymatic degradation by glycosidases, which are also present in *A. oryzae* medium-filtrate (Kanaya and Uchida, 1981). In *P. inflata*, S₃ protein, which normally has a single glycan chain, was mutagenized and gave rise to S₃(N29D), a nonglycosylated protein. The ability of S₃(N29D) protein to gain complete inhibition of S₃ pollen indicates that the carbohydrate moiety is not necessarily required for the self-incompatibility reaction (Karunanandaa et al., 1994). In *Lycopersicon peruvianum* (L.) Mill. the S₃-RNase has a single N-glycosylation site to which one of three n-glycans is attached (Parry et al., 1998). The exact biological role of the glycans is not understood. The molecular model of Parry et al. (1998) suggest that the glycans are not involved in determining the self-incompatibility phenotype. It is possible the glycans play a role in the maintenance of glycoprotein stability and in improving their solubility (Rademacher et al., 1988; Parry et al., 1998).

In the present work, RNase B1 had a clear inhibitory effect on pollen germination and tube growth, in vitro as well as in vivo. The optimal temperature for RNase B1 activity is 60 °C, however, at 25 °C the enzyme still possesses 25% of its activity. In both peach and tangerine the appearance of the RNase-treated pollen tubes resembled the morphological features described in incompatible pollen tubes for *Petunia hybrida* Hort. Vilm.-Andr. (Herrero and Dickinson, 1980) and *Papaver rhoeas* L. (Franklin-Tong and Franklin, 1992). The pollen tubes were short, burst easily, had irregularities in wall deposition, and lacked a definite growth orientation. In gametophytic self-incompatible plants, the S-RNases have been found to share homology with RNase T₂, RNase Rh, and RNase M (Haring et al, 1990; McClure et al., 1989). In previous work we described intensive callose deposition in RNase-inhibited pollen tubes in calamondin in response to stigmatic RNase and RNase T1 (Roiz et al., 1995). The inhibitory effect of RNase B1 on pollen tube growth appears to resemble that of the other RNases.

In nectarine, RNase B1 treatment reduces fruit set, indicating that its ability to inhibit pollen tube growth in the style may lead to a decrease in the fertilization rate. During the first month following blooming period, fruitlets born on RNase-treated branches were smaller and showed larger falling ratio than the controls. The fact that RNase B1 affected the embryos viability, may indicate its potential to interfere with fertilization.

In conclusion, *A. niger* B1 was found to be an efficient source of RNase. The RNase B1 inhibited pollen germination and pollen tube growth nonspecifically, but pollen tubes of treated pollen

displayed morphological traits similar to those in gametophytic self-incompatibility. Much work is still needed to formulate RNase B1 as an effective control agent. However, based on this work the potential of its biological effect is clear, with its additional advantage of being environmentally safe.

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