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# Synergistic Enhancement of the Antifungal Activity of Wheat and Barley Thionins by Radish and Oilseed Rape 2S Albumins and by Barley Trypsin Inhibitors[1]

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**Although thionins and 2S albumins are generally considered as storage proteins, both classes of seed proteins are known to inhibit the growth of pathogenic fungi. We have now found that the wheat (*Triticum aestivum* L.) or barley (*Hordeum vulgare* L.) thionin concentration required for 50% inhibition of fungal growth is lowered 2- to 73-fold when combined with 2S albumins (at sub- or noninhibitory concentrations) from radish (*Raphanus sativus* L.) or oilseed rape (*Brassica napus* L.). Furthermore, the thionin antifungal activity is synergistically enhanced (2- to 33-fold) by either the small subunit or the large subunit of the radish 2S albumins. Three other 2S albumin-like proteins, the barley trypsin inhibitor and two barley Bowman-Birk-type trypsin inhibitor isoforms, also act synergistically with the thionins (2- to 55-fold). The synergistic activity of thionins combined with 2S albumins is restricted to filamentous fungi and to some Gram-positive bacteria, whereas Gram-negative bacteria, yeast, cultured human cells, and erythrocytes do not show an increased sensitivity to thionin/albumin combinations (relative to the sensitivity to the thionins alone). Scanning electron microscopy and measurement of $K^+$ leakage from fungal hyphae revealed that 2S albumins have the same mode of action as thionins, namely the permeabilization of the hyphal plasmalemma. Moreover, 2S albumins and thionins act synergistically in their ability to permeabilize fungal membranes.**

Plant seeds are able to survive and resist environmental damage for several months. Essential to this resting state is a low water content and thus a low metabolic activity, which resumes after rehydration and germination of the seeds. Germination also involves the mobilization of storage compounds, e.g. storage proteins, that are sequestered in specialized seed tissues such as cotyledons and endosperm. Much of our current knowledge on seed proteins stems from studies on storage proteins, i.e. proteins that accumulate to high levels during seed maturation and are rapidly degraded during germination (Higgins, 1984). Well-known examples of such storage proteins are the 2S albumins, which have been detected in seeds of many species including radish (Terras et al., 1992b) and oilseed rape (Ericson et al., 1986; Terras et al., 1993).

Other seed proteins are thought to play a role in the protection of resting seeds or seedlings against insect attack or microbial infection. Examples of such proteins are chitinases and glucanases (Boller, 1988; Leah et al., 1991), ribosome-inactivating proteins (Leah et al., 1991), chitin-binding lectins (Raikhel et al., 1993), or more recently discovered antimicrobial proteins (Hejgaard et al., 1991; Broekaert et al., 1992; Cammue et al., 1992; Duvick et al., 1992; Terras et al., 1992a, 1992b, 1993; Vigers et al., 1992).

In a number of cases, however, the distinction between storage function and defense function is vague, and many authors have proposed a dual role for storage proteins. For instance, cereals contain proteins homologous to 2S albumins from dicotyledons, but which additionally display inhibiting activities against proteases or α-amylases (Garcia-Olmedo et al., 1987). Such enzyme inhibitors may decrease the digestibility of seeds for insects and vertebrates (Ryan, 1989). More recently, we have demonstrated that 2S albumins from seeds of a number of Brassicaceae species inhibit the growth of several plant pathogenic fungi (Terras et al., 1992b, 1993). Thionins are another class of abundant endosperm proteins from cereals that have proven antifungal and antibacterial activities and are cytotoxic to various animal cells (Garcia-Olmedo et al., 1989; Bohlmann and Apel, 1991).

In the present paper we report that two types of seed proteins with presumed storage functions, namely 2S albumins and thionins, act synergistically with respect to their antifungal activities. Moreover, we present evidence that both proteins affect the permeability of fungal membranes and

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Abbreviations: α-PT, α-purothionin; *Bn*-2S, 2S albumins from *Brassica napus* seeds; *Hv*-Ti, trypsin inhibitor from *Hordeum vulgare* grains; $IC_{50}$, protein concentration required for 50% growth inhibition; LS, *Rs*-2S large subunit; PDB, potato dextrose broth; ppm, parts per million; *Rs*-2S3, *Raphanus sativus* 2S albumin fraction 3; SS, *Rs*-2S small subunit.

that combinations of both proteins result in an enhancement of membrane permeabilization. These data support the view that many seed proteins may protect seeds from microbial infestation in addition to their role as temporary carbon, nitrogen, and sulfur stores.

## MATERIALS AND METHODS

### Antibiotic Activity Assays

Antifungal, antibacterial, and antiyeast activities of a protein sample were assayed by microspectrophotometry (Broekaert et al., 1990) as described previously (Terras et al., 1992b). Briefly, in a well of a 96-well microplate, 20 $\mu$L of the protein sample was mixed with 80 $\mu$L of the appropriate growth medium (half-strength PDB [Difco, Detroit, MI] for fungi; 1% [w/v] tryptone, 0.5% [w/v] low-melting-point agarose for bacteria; and half-strength PDB, 0.5% [w/v] low-melting-point agarose for yeast) containing fungal spores, bacterial cells, or yeast cells. $A_{595}$ served as a measure for microbial growth (Terras et al., 1992b). The following strains were used: *Alternaria brassicola* (MUCL 20297), *Ascochyta pisi* (MUCL 20164), *Botrytis cinerea* (MUCL 30158), *Fusarium culmorum* (IMI 180420), *Verticillium dahliae* (MUCL 6963), *Bacillus megaterium* (ATCC 13632), *Sarcina lutea* (ATCC 9342), *Escherichia coli* (strain HB101), *Erwinia carotovora* (strain 3912), and *Saccharomyces cerevisiae* (strain Sp1).

Toxicity for human skin muscle fibroblasts was assayed by vital staining with neutral red as described (Terras et al., 1992b), whereas hemolytic activity was assayed by spectrophotometric monitoring of hemoglobin released from human erythrocytes (Terras et al., 1992b).

### Protein Purification

Thionins (purothionins from *Triticum aestivum* [wheat] grains or hordothionins from *Hordeum vulgare* [barley] grains) were purified by the method of Redman and Fisher (1969) followed by a final purification step wherein the thionin samples were loaded onto a reversed-phase chromatography column (Pep-S $C_2/C_{18}$ 15-$\mu$m porous silica, 25 × 0.93 cm; Pharmacia) equilibrated with 0.1% (v/v) TFA at a flow rate of 5 mL/min. A linear gradient (of 200 mL) to 40% (v/v) acetonitrile/0.1% (v/v) TFA was applied to desorb the bound proteins. The thionins eluted at approximately 34% (v/v) acetonitrile/0.1% (v/v) TFA. Fractions containing the thionins were vacuum dried to remove the solvents and redissolved in Milli-Q water. The $\alpha$-PT preparation used in the experiments described in this study is $\alpha$2-PT.

We isolated 2S albumins from *Raphanus sativus* L. and *Brassica napus* L. as described previously (Terras et al., 1992b, 1993). Trypsin inhibitors from *H. vulgare* were purified according to the procedure of Mikola and Suolinna (1969), which was modified as follows: 1 kg of barley flour was mixed with 2 L of distilled water and the suspension was acidified to pH 4.5 by addition of 1 M acetic acid. After 2 h at 5°C without stirring, the solid material was removed by centrifugation (15 min at 7000*g*) and the supernatant was left overnight at 5°C. Material precipitating after boiling for 15 min was removed by centrifugation (15 min at 7000*g*) and solid $(NH_4)_2SO_4$ was added to the supernatant to 40% relative saturation. Proteins aggregating under these conditions were collected by centrifugation (30 min at 7000*g*) and the pellets were suspended in a total of 300 mL of distilled water. The insoluble precipitate was removed by centrifugation (30 min at 7000*g*) and the supernatant was extensively dialyzed against distilled water. After dialysis, 0.5 M Mes at pH 6 was added to a final concentration of 50 mM. This extract was subsequently loaded on a cation-exchange column (S-Sepharose high performance, 10 × 1.6 cm; Pharmacia) in equilibrium with 50 mM Mes, pH 6. The bound fraction was eluted with a linear gradient of 500 mL from 0 to 1 M NaCl in 50 mM Mes (pH 6) at a flow rate of 2.5 mL/min. The eluate was collected in 20-mL fractions.

A first peak of trypsin-inhibiting activity (measured by the inhibition of hydrolysis of benzoyl-DL-Arg-*p*-nitroanilide by trypsin) eluted between 240 and 320 mM NaCl. This material was further purified by reversed-phase chromatography (Pep-S $C_2/C_{18}$ 15-$\mu$m porous silica, 25 × 0.93 cm; Pharmacia) yielding *Hv*-Ti1 (*H. vulgare* trypsin inhibitor 1) eluting at approximately 35% (v/v) acetonitrile/0.1% (v/v) TFA under the conditions described above for the purification of the thionins. Its identity was unambiguously determined by automated amino acid sequencing: the $NH_2$-terminal 11 amino acids (FGDSCAPGDAL) were identical to the sequence of the barley trypsin inhibitor described by Odani et al. (1983).

A second peak of trypsin-inhibiting activity eluted between 400 and 500 mM NaCl. By reversed-phase chromatography (under the conditions described above), two inhibitor peaks were obtained: *Hv*-Ti2 (eluting at 14% [v/v] acetonitrile/0.1% [v/v] TFA) and *Hv*-Ti3 (eluting at 16% [v/v] acetonitrile/0.1% [v/v] TFA). $NH_2$-terminal amino acid sequencing revealed that *Hv*-Ti2 (AGKKRPWKCC) and *Hv*-Ti3 (AGKKIPWKCC) were isoforms with strong sequence homology with WGI1, a Bowman-Birk-type trypsin inhibitor from wheat germ (Odani et al., 1986), the corresponding $NH_2$-terminal region of the latter being AAKKRPWKCC.

### Oxido-Shuffling and Thiol Dosage

Reoxidation of disulfide bonds was performed using the oxido-shuffling system consisting of 0.3 mM GSSH and 3 mM GSH according to Jaenicke and Rudolph (1990). Free thiol groups were quantitated as described (Creighton, 1990) using the Ellman assay based on the release of nitrothiobenzoate upon reaction of a free thiol with dithionitrobenzoate.

### Scanning EM

Glass coverslips (12 mm diameter) were washed for 5 min in 25% (v/v) sulfuric acid, rinsed with bidistilled water, and finally incubated for 30 min at 37°C in a 0.01% (w/v) solution of poly-L-Lys (mol wt approximately 10,700; Sigma). The poly-L-Lys-coated coverslips were placed on the bottom of a 24-well microplate and covered with 0.5 mL of a suspension of fungal spores ($2 \times 10^4$ spores/mL in half-strength PDB) in the absence or presence of antifungal proteins. The fungus was grown for 36 h at 25°C. Hyphae attached to the coverslips were fixed for 2 h in fixing solution (0.1 M cacodylate, 2% [v/v] formaldehyde, 2.5% [v/v] glutaraldehyde, pH 6.8)

**Table I.** *Antifungal activity of α-PT, Rs-2S3, and Bn-2S*

$IC_{50}$ values after 48 h of incubation were determined from the dose-response curves (percent growth inhibition versus protein concentration).

| Fungus | $IC_{50}$ Values | | | | | |
|---|---|---|---|---|---|---|
| | Medium A[a] | | | Medium B[b] | | |
| | α-PT | *Rs*-2S3 | *Bn*-2S | α-PT | *Rs*-2S3 | *Bs*-2S |
| | μg/mL | | | μg/mL | | |
| *A. brassicola* | 4 | 10 | 45 | 11 | >1000 | >1000 |
| *A. pisi* | 8 | 75 | 102 | 12 | >1000 | >1000 |
| *F. culmorum* | 4 | 35 | 95 | 11 | >1000 | >1000 |
| *V. dahliae* | 2 | 33 | 45 | 8 | >1000 | >1000 |

[a] Medium A, Half-strength PDB. [b] Medium B, Half-strength PDB with 1 mM $CaCl_2$ and 50 mM KCl.

and washed with 0.125 M cacodylate (pH 6.8). The samples were sequentially dehydrated in 30, 50, 70, 90, and 100% (v/v) ethanol and critical point dried with $CO_2$. The coverslips were then mounted on carbon filters, sealed with silver paint, and covered with gold in a sputtercoater (Polaran Instruments, Inc., Hatfield, PA). The samples were viewed in a Philips PSEM500 scanning electron microscope operated at 20 kV. These experiments were performed with *B. cinerea* as a test fungus. *F. culmorum* could not be used because hyphae were washed off from the coverslips during the fixation step under the conditions described above.

### Atomic Absorption Spectrometry

*F. culmorum* was grown for 48 h at 25°C at an inoculum density of $10^4$ spores/mL in 50-mL Erlenmeyer flasks containing 10 mL of half-strength PDB. The fungal hyphae were collected on No. 2 Whatman filter paper, washed with bidistilled water, and resuspended in 10 mL of an aqueous solution of 0.1% (w/v) Glc containing 25 μM $CaCl_2$ and 25 μM $MgCl_2$ and supplemented with the appropriate antifungal compounds. After 1.5 h of incubation in this medium, $K^+$ efflux in the medium was measured with an atomic absorption spectrometer (Varian SpectrAA-10 Plus) at 766.5 nm with a bandwidth of 1 nm.

## RESULTS

### Synergistic Enhancement of Thionin Antifungal Activity by Brassicaceae 2S Albumins

In a first experiment, dose-response curves (percent growth inhibition versus protein concentration) of α-PT, *Rs*-2S3 (Terras et al., 1992b), and *Bn*-2S (a mixture of different isoforms; Terras et al., 1993) were determined using four different fungi. This was done separately in half-strength PDB (medium A) and half-strength PDB supplemented with 1 mM $CaCl_2$ and 50 mM KCl (medium B). From these curves, the $IC_{50}$ values were derived (Table I). The antifungal activity of α-PT is only slightly affected by the added salts, whereas *Rs*-2S3 and *Bn*-2S are active in medium A but inactive in medium B at concentrations up to 1000 μg/mL.

In a second experiment, *Rs*-2S3 or *Bn*-2S were added at different constant subinhibitory concentrations to serial dilutions of α-PT. Again, the $IC_{50}$ values were determined and the synergistic antifungal effect of the two proteins (synergism factor) is expressed as the ratio of the $IC_{50}$ value of α-PT alone over the $IC_{50}$ value of α-PT in combination with *Rs*-2S3 or *Bn*-2S (Table II). An example of the dose-response curves (for α-PT, *Rs*-2S3, and combinations of both) obtained using *F. culmorum* as a test organism is shown in Figure 1. In medium A, synergism factors ranging from 3 to 9 or from 4 to 18 (depending on the fungus) were obtained when *Rs*-2S3 or *Bn*-2S, respectively, were added at a constant concentration of 10 μg/mL. In medium B, *Rs*-2S3 or *Bn*-2S were added to α-PT dilution series at three different subinhibitory con-

**Table II.** *Synergistic enhancement of α-PT antifungal activity by Rs-2S3 or Bn-2S*

Concentrations of α-PT required for $IC_{50}$ in the presence of different subinhibitory concentrations of *Rs*-2S3 or *Bn*-2S were determined from the dose-response curves after 48 h of incubation. Synergism factors (rounded to the nearest integer) are expressed as the ratio of the α-PT $IC_{50}$ over the $IC_{50}$ of the combination of α-PT with *Rs*-2S3 or *Bn*-2S.

| Fungus | Test Protein | $IC_{50}$ Values (μg/mL) of α-PT (and Synergism Factor) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Medium A[a] | | Medium B[b] | | | |
| | | Test protein (μg/mL) | | Test protein (μg/mL) | | | |
| | | 0 | 10 | 0 | 10 | 50 | 250 |
| *A. brassicola* | *Rs*-2S3 | 4 | 0.86 (5×) | 11 | 6.0 (2×) | 0.45 (25×) | 0.25 (44×) |
| | *Bn*-2S | 4 | 0.22 (18×) | 11 | 2.0 (5×) | 0.32 (34×) | 0.15 (73×) |
| *A. pisi* | *Rs*-2S3 | 8 | 2.50 (3×) | 12 | 5.5 (2×) | 1.00 (12×) | 0.80 (14×) |
| | *Bn*-2S | 8 | 2.00 (4×) | 12 | 7.0 (2×) | 3.00 (4×) | 1.50 (8×) |
| *F. culmorum* | *Rs*-2S3 | 4 | 0.45 (9×) | 11 | 3.0 (4×) | 0.40 (27×) | 0.40 (27×) |
| | *Bn*-2S | 4 | 0.25 (16×) | 11 | 5.0 (2×) | 1.00 (11×) | 1.50 (7×) |
| *V. dahliae* | *Rs*-2S3 | 2 | 0.40 (5×) | 8 | 1.1 (7×) | 0.40 (20×) | 0.40 (20×) |
| | *Bn*-2S | 2 | 0.15 (13×) | 8 | 0.7 (11×) | 0.22 (36×) | 0.20 (40×) |

[a] Medium A, Half-strength PDB. [b] Medium B, Half-strength PDB with 1 mM $CaCl_2$ and 50 mM KCl.

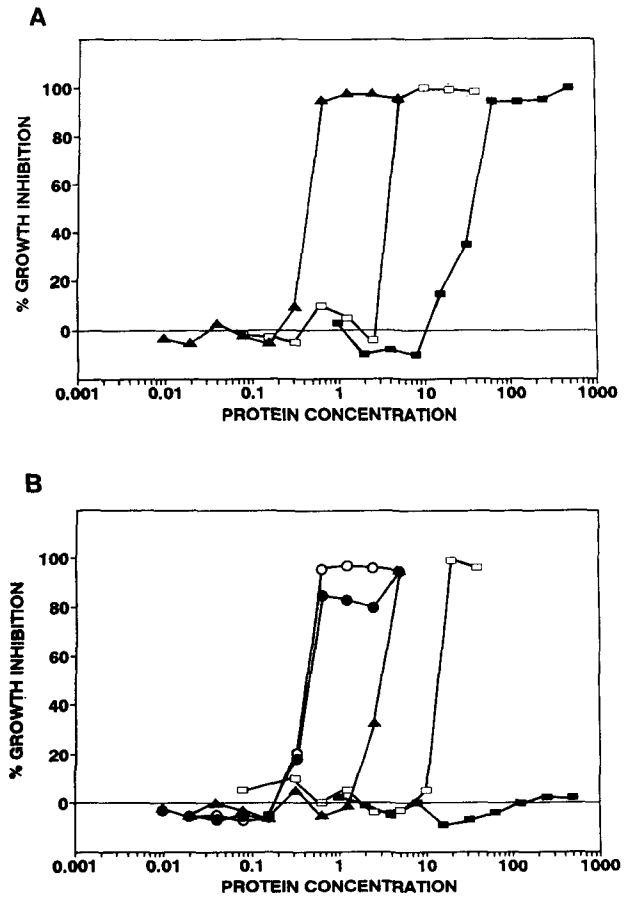


**Figure 1.** Dose-response curves determined for *F. culmorum* after a 48-h incubation period. A, In half-strength PDB: (■), serial dilution of *Rs*-2S3; (□), serial dilution of α-PT; (▲), serial dilution of α-PT in the presence of 10 μg/mL *Rs*-2S3. B, In half-strength PDB with 1 mM $CaCl_2$ and 50 mM KCl: (■), serial dilution of *Rs*-2S3; (□), serial dilution of α-PT; (▲), serial dilution of α-PT in the presence of 10 μg/mL *Rs*-2S3; (○), serial dilution of α-PT in the presence of 50 μg/mL *Rs*-2S3; (●), serial dilution of α-PT in the presence of 250 μg/mL *Rs*-2S3. Protein concentration is in μg/mL.

centrations: 10, 50, and 250 μg/mL. In this medium, a clear increase of the synergism factors is observed when the *Rs*-2S3 or *Bn*-2S concentration is raised from 10 μg/mL (synergism factors from 2–7 or from 2–11, respectively) to 50 μg/mL (synergism factors from 11–27 or from 4–36, respectively). With the exception of *A. brassicola*, no further substantial increase of the synergism factors is seen when the *Rs*-2S3 or *Bn*-2S concentration is raised from 50 to 250 μg/mL.

Comparable synergism factors were also obtained upon addition of equal concentrations of any of the other four *Rs*-2S isoform fractions (*Rs*-2S1, *Rs*-2S2, *Rs*-2S4, and *Rs*-2S5; fractionation of the radish 2S albumins is based on differences in charge, see Terras et al., 1992b) or of a mixture of the five *Rs*-2S isoforms (results not shown). Moreover, similar results were obtained when α-PT was substituted by β-PT or by either the α- or β-hordothionins (the thionins from barley grains) in antifungal activity tests performed on *F. culmorum* (results not shown).

## Antifungal Activity of Radish 2S Albumin Subunits and Synergism with Thionins

2S albumins are composed of two different subunits that are derived from a single precursor polypeptide and are linked together by disulfide bridges (Krebbers et al., 1988). Therefore, it was worthwhile to investigate which subunit is responsible for enhancing the thionin antifungal activity. SS and LS were prepared from *Rs*-2S3 after cleavage of the disulfide bonds as described (Terras et al., 1992b). DTT was added at a final concentration of 0.25 mM to prevent cystine formation, which is reported to occur spontaneously in SS (Monsalve et al., 1991). Additionally, a fraction of the in SS and the LS preparation was intentionally reoxidized by oxidoshuffling. After purification by reversed-phase chromatography (not shown), the residual free thiol content (compared with the reduced forms in the absence of DTT) was determined as 9% for the reoxidized SS and 22% for the reoxidized LS preparations.

Antifungal activity against *F. culmorum* of all protein preparations was determined and expressed as $IC_{50}$ values in Table III. DTT at 0.05 mM, the highest concentration applied in the fungal inhibition assay (because samples are diluted 5-fold, see "Materials and Methods"), does not inhibit the growth of *F. culmorum*. Whether reduced or oxidized, only the SS exerts antifungal activity ($IC_{50}$ values of 14 or 32 μg/mL, respectively), which is abolished by addition of 1 mM $CaCl_2$ and 50 mM KCl to the assay medium.

Subsequently, reoxidized SS and LS were assessed for possible enhancement of the α-PT antifungal activity in the same way as described above for the native *Rs*-2S3 (Table IV). Synergistic antifungal activity was observed for both reoxidized SS (2- to 33-fold) and reoxidized LS (2- to 13-fold) and both in the medium with or without 1 mM $CaCl_2$ and 50 mM KCl. However, the synergism factors under the high-ionic strength conditions are not as pronounced as those obtained for native *Rs*-2S3.

**Table III.** *Antifungal activity against F. culmorum of reduced small and large subunits (SSre and LSre) and reoxidized small and large subunits (SSox and LSox) derived from radish 2S albumins*

$IC_{50}$ values after 48 h of incubation were determined from the dose-response curves.

| Protein | $IC_{50}$ Values | |
|---|---|---|
| | Medium A[a] | Medium B[b] |
| | μg/mL | |
| SSre | 14 | >200 |
| SSox | 32 | >200 |
| LSre | >400 | >400 |
| LSox | >400 | >400 |

[a] Medium A, Half-strength PDB. [b] Medium B, Half-strength PDB with 1 mM $CaCl_2$ and 50 mM KCl.

**Table IV.** *Synergistic enhancement of α-PT antifungal activity against F. culmorum by reoxidized small and large subunit fractions (SSox and LSox) of the radish 2S albumins*

$IC_{50}$ values in the presence of different subinhibitory concentrations of the reoxidized small and large subunit were determined from the dose-response curves after 48 h of incubation. Synergism factors (rounded to the nearest integer) are expressed as the ratio of the α-PT $IC_{50}$ over the $IC_{50}$ of the combination of α-PT with SSox or LSox.

| Test Protein | $IC_{50}$ Values (μg/mL) of α-PT (and Synergism Factor) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Medium A[a] | | | Medium B[b] | | | | |
| | Test protein (μg/mL) | | | Test protein (μg/mL) | | | | |
| | 0 | 10 | 20 | 0 | 10 | 20 | 50 | 400 |
| SSox | 4 | 0.12 (33×) | N.D.[c] | 11 | 5.2 (2×) | N.D. | 0.8 (14×) | N.D. |
| LSox | 4 | N.D. | 0.32 (13×) | 11 | N.D. | 6 (2×) | N.D. | 0.7 (12×) |

[a] Medium A, Half-strength PDB. [b] Medium B, Half-strength PDB with 1 mM $CaCl_2$ and 50 mM KCl. [c] N.D., Not determined.

### Synergistic Antifungal Activity between Thionins and 2S Albumin-Like Trypsin Inhibitors

To answer the question whether the synergistic enhancement of the α-PT antifungal activity is a feature unique to the Brassicaceae 2S albumins, we purified the 2S-like trypsin inhibitor from barley (hereafter referred to as *H. vulgare* trypsin inhibitor 1, abbreviated to *Hv*-Ti1). In contrast to the Brassicaceae 2S albumins, *Hv*-Ti1 is composed of a single polypeptide chain (Odani et al., 1983). During the *Hv*-Ti1 purification process, we also purified two trypsin-inhibitor isoforms (*Hv*-Ti2 and *Hv*-Ti3; see "Materials and Methods") of the Bowman-Birk type known to occur in barley (Boisen and Djurtoft, 1982) as well as in wheat grains (Odani et al., 1986). It is known that Bowman-Birk protease inhibitors show a weak homology to the LS domain of 2S albumins (Sharief and Li, 1982).

Antifungal activity of *Hv*-Ti1 (see also Terras et al., 1993), *Hv*-Ti2, and *Hv*-Ti3 are given in Table V. In medium A, *Hv*-Ti1 has a rather weak antifungal activity. Surprisingly, *Hv*-Ti2 and *Hv*-Ti3 have antifungal activities similar to that of Brassicaceae 2S albumins. From the data in Tables I and V, it can be concluded that cation sensitivity is a common characteristic of the antifungal activity of 2S albumins and 2S albumin-like trypsin inhibitors, since addition of 1 mM $CaCl_2$ and 50 mM KCl to half-strength PDB suppresses the activity of these proteins, even at concentrations as high as 1 mg/mL.

Another common characteristic of these proteins, besides their intrinsic antifungal activity, seems to be the synergistic enhancement of the α-PT antifungal activity. Synergism factors of combinations of α-PT and either of the trypsin-inhibitor preparations are given in Table VI. The synergism factors obtained for the α-PT/*Hv*-Ti combinations are generally lower than those for the α-PT/Brassicaceae 2S albumin combinations. In the medium supplemented with 1 mM $CaCl_2$ and 50 mM KCl, synergism factors from 7- to 55-fold are displayed at *Hv*-Ti levels of 250 μg/mL, whereas the Brassicaceae 2S albumins give comparable synergism factors at a concentration of 50 μg/mL. As for the Brassicaceae 2S albumins, substitution of α-PT by β-PT, α-hordothionin, or β-hordothionin does not affect the synergism with the barley protease inhibitors on *F. culmorum* (results not shown).

### Enhancement of Thionin Cytotoxic Activity by 2S Albumins Is Restricted to Fungi and Some Gram-Positive Bacteria

In view of the enhancement of the α-PT antifungal activity by different 2S albumins, it could be expected that the latter proteins are able to enhance other activities exerted by α-PT against bacteria, yeast, cultured human cells, and erythrocytes. The growth of the Gram-positive bacterium *B. megaterium* is known to be affected by *Rs*-2S ($IC_{50}$ value of 10 μg/mL for *Rs*-2S3; Terras et al., 1992b), as well as by α-PT ($IC_{50}$ value of 1 μg/mL; Cammue et al., 1992). Addition of salts (1 mM $CaCl_2$, 50 mM KCl) to the test medium (consisting of 1% [w/v] tryptone and 0.5% [w/v] agarose) abolishes the antibacterial activity of *Rs*-2S3 but not that of α-PT ($IC_{50}$ value of 2 μg/mL). In this medium with added salts, however, noninhibitory concentrations of *Rs*-2S3 potentiate the antibacterial activity of α-PT 4-fold (*Rs*-2S3 at 10 μg/mL), 16-fold (*Rs*-2S3 at 50 μg/mL), or 32-fold (*Rs*-2S3 at 250 μg/mL). *S. lutea*, another Gram-positive bacterium, and *E. coli* (Gram-negative) are inhibited by α-PT itself but not by *Rs*-2S3, whereas *E. carotovora* (Gram-negative) is inhibited by *Rs*-2S3 but not by α-PT (Terras et al., 1992b). However, any combination of the two proteins failed to result in synergistically enhanced antibacterial activity. Furthermore, the antiyeast activity of α-PT is not enhanced by *Rs*-2S3, which by itself is unable to inhibit growth of *S. cerevisiae* cells (Terras et al., 1992b).

Fifty percent of cultured human skin-muscle fibroblasts are killed by α-PT at a concentration of 25 μg/mL. Again, *Rs*-2S3 at 250 μg/mL (not toxic for this cell type; Terras et al., 1992b) is unable to lower the $IC_{50}$ value of α-PT. A similar observation was made for the hemolytic effect of α-PT on human erythrocytes, which also was not enhanced by the presence of *Rs*-2S3 at 250 μg/mL.

**Table V.** *Antifungal activity of trypsin inhibitors isolated from grains of H. vulgare (Hv-Ti1, Hv-Ti2, and Hv-Ti3)*

$IC_{50}$ values after 48 h of incubation were determined from the dose-response curves.

| Fungus | $IC_{50}$ Values | | | | | |
|---|---|---|---|---|---|---|
| | Medium A[a] | | | Medium B[b] | | |
| | *Hv*-Ti1 | *Hv*-Ti2 | *Hv*-Ti3 | *Hv*-Ti1 | *Hv*-Ti2 | *Hv*-Ti3 |
| | μg/mL | | | μg/mL | | |
| *A. brassicola* | 280 | 55 | 42 | >1000 | >500 | >500 |
| *A. pisi* | >1000 | 105 | 90 | >1000 | >500 | >500 |
| *F. culmorum* | 910 | 110 | 90 | >1000 | >500 | >500 |
| *V. dahliae* | 350 | 65 | 50 | >1000 | >500 | >500 |

[a] Medium A, Half-strength PDB. [b] Medium B, Half-strength PDB with 1 mM $CaCl_2$ and 50 mM KCl.

## 2S Albumins and Thionins Permeabilize Hyphal Membranes

Since the antifungal activity of 2S albumins was discovered only very recently (Terras et al., 1992b, 1993), nothing is known about their possible mode of action. To investigate this, spores of *B. cinerea* were allowed to germinate in the presence of *Rs*-2S3 at 50 μg/mL (this is the $IC_{50}$ value of *Rs*-2S3 against *B. cinerea*). After a 36-h incubation, the emerged hyphae were examined by scanning EM. Figure 2C shows a typical picture of these hyphae. Compared with the control hyphae (Fig. 2A), there seems to be no apparent alteration of the cell wall structure. Thus, a possible effect of *Rs*-2S3 on cell wall synthesis is unlikely. However, at least 25% of the observed hyphae treated with *Rs*-2S3 extrude cytoplasmic material at positions close to the hyphal tips. A similar effect is observed for hyphae treated with α-PT at 50 μg/mL (this is the $IC_{50}$ value of α-PT against *B. cinerea*; Fig. 2B).

To further substantiate these observations suggesting membrane permeabilization, the leakage of $K^+$ from *F. culmorum* hyphae was quantitated by atomic absorption spectrometry. As depicted in Figure 3A, there is a correlation between the extent of $K^+$ leakage and growth inhibition caused by α-PT or *Rs*-2S3. Taken together, these data suggest a similar mode of action for α-PT and *Rs*-2S3, namely permeabilization of fungal plasmalemma close to the hyphal tip.

To address the question whether this could also explain the observed synergistic antifungal activity between these proteins, the $K^+$ leakage caused by combinations of α-PT and *Rs*-2S3 was determined at concentrations at which neither of the two proteins alone results in $K^+$ leakage. Figure 3B clearly illustrates that such combinations indeed efficiently permeabilize hyphal membranes. For instance, the same extent of $K^+$ leakage caused by either α-PT alone at 12.5 μg/mL (Fig. 3A) or *Rs*-2S3 alone at 50 μg/mL (Fig. 3A) can be reached

**Table VI.** *Synergistic enhancement of α-PT antifungal activity of Hv-Ti1, Hv-Ti2, and Hv-Ti3*

$IC_{50}$ values in the presence of different subinhibitory concentrations of one of the inhibitor preparations were determined from the dose-response curves. Synergism factors (rounded to the nearest integer) are expressed as the ratio of the α-PT $IC_{50}$ over the $IC_{50}$ of the combination of α-PT with one of the inhibitor proteins.

| Fungus | Test Protein | $IC_{50}$ Values (μg/mL) of α-PT (and Synergism Factor) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Medium A[a] | | Medium B[b] | | | |
| | | Test protein (μg/mL) | | Test protein (μg/mL) | | | |
| | | 0 | 10 | 0 | 10 | 50 | 250 |
| *A. brassicola* | *Hv*-Ti1 | 4 | 0.20 (20×) | 11 | 2.5 (4×) | 1.20 (9×) | 0.35 (31×) |
| | *Hv*-Ti2 | 4 | 0.80 (5×) | 11 | 8.0 (1×) | 2.50 (4×) | N.D.[c] |
| | *Hv*-Ti3 | 4 | 0.28 (14×) | 11 | 8.0 (1×) | 1.50 (7×) | 0.20 (55×) |
| *A. pisi* | *Hv*-Ti1 | 8 | 3.00 (3×) | 12 | 10.0 (1×) | 6.00 (2×) | 1.20 (10×) |
| | *Hv*-Ti2 | 8 | 7.00 (1×) | 12 | 11.0 (1×) | 9.00 (1×) | 1.40 (8×) |
| | *Hv*-Ti3 | 8 | 4.00 (2×) | 12 | 12.0 (1×) | 9.00 (1×) | 1.20 (10×) |
| *F. culmorum* | *Hv*-Ti1 | 4 | 0.40 (10×) | 11 | 10.0 (1×) | 7.00 (2×) | 0.90 (12×) |
| | *Hv*-Ti2 | 4 | 1.50 (3×) | 11 | 10.0 (1×) | 2.50 (4×) | 1.10 (10×) |
| | *Hv*-Ti3 | 4 | 0.90 (4×) | 11 | 10.0 (1×) | 2.50 (4×) | 1.20 (9×) |
| *V. dahliae* | *Hv*-Ti1 | 2 | 0.14 (14×) | 8 | 2.8 (3×) | 0.90 (9×) | 0.40 (20×) |
| | *Hv*-Ti2 | 2 | 0.60 (3×) | 8 | 3.0 (3×) | 0.90 (9×) | 1.10 (7×) |
| | *Hv*-Ti3 | 2 | 0.30 (7×) | 8 | 1.5 (5×) | 0.40 (20×) | 0.80 (10×) |

[a] Medium A, Half-strength PDB. [b] Medium B, Half-strength PDB with 1 mM $CaCl_2$ and 50 mM KCl. [c] N.D., Not determined.

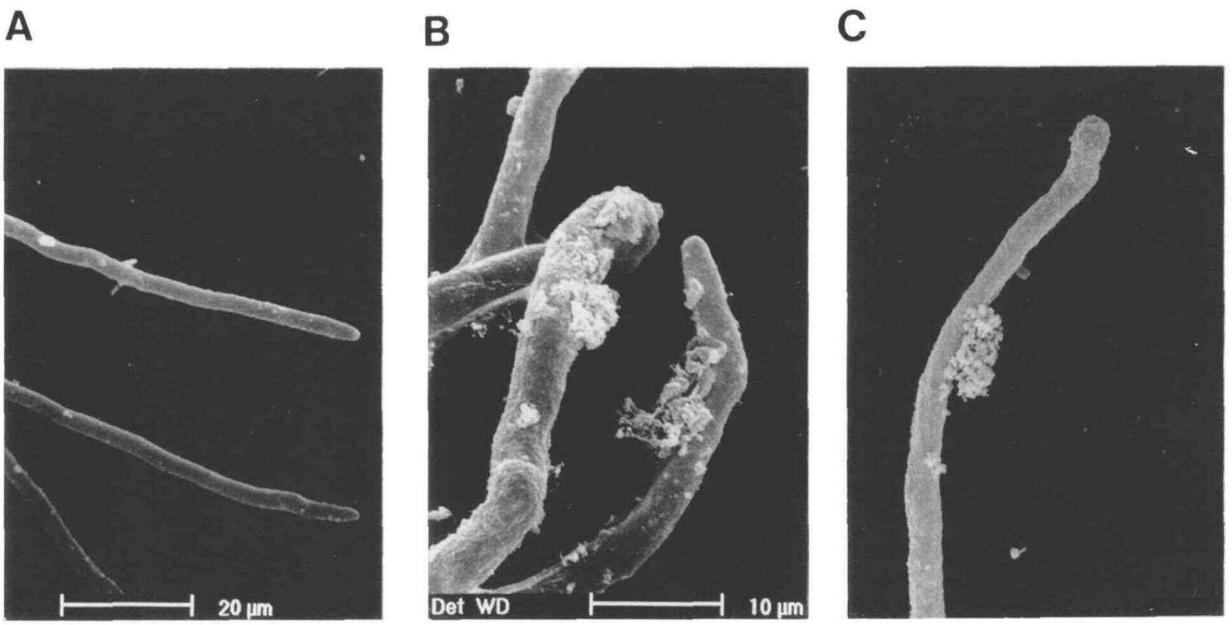


**Figure 2.** Scanning electron micrographs of *B. cinerea* hyphae grown for 36 h in the presence of A, water (control), bar corresponds to 20 μm; B, α-PT at 50 μg/mL, bar corresponds to 10 μm; and C, *Rs*-2S3 at 50 μg/mL, same magnification as B.

when combining α-PT at 0.5 μg/mL with *Rs*-2S3 at 10 μg/mL (Fig. 3B).

## DISCUSSION

We previously showed that the 14-kD heterodimeric 2S albumins from Brassicaceae seeds are inhibitors of fungal growth and that their activity is strongly affected by the presence of inorganic cations in the assay medium (Terras et al., 1992b, 1993). Although much weaker, antifungal properties are also exhibited by the trypsin inhibitor from barley (*Hv*-Ti1). *Hv*-Ti1 is a single, 13-kD polypeptide with an $NH_2$-terminal domain resembling the SS of 2S albumins and a COOH-terminal part homologous to the LS of 2S albumins (Odani et al., 1983). Surprisingly, two Bowman-Birk-type trypsin inhibitors from barley (*Hv*-Ti2 and *Hv*-Ti3) also inhibit fungal growth to a similar extent as the 2S albumins. It has been observed previously that Bowman-Birk trypsin inhibitors show weak homology to the LS of 2S albumins (Sharief and Li, 1982). Antifungal activity does not seem to be a general property of this class of protease inhibitors since we have found that the Bowman-Birk trypsin inhibitor from soybean is not active against fungi, including those used in the present study (Terras et al., 1992b). Separation of the radish 2S albumin into its subunits after reduction of the intersubunit disulfide bridges revealed that the small subunit, but not the large subunit, displays antifungal activity. However, lack of activity of the large subunit preparation may be due to conformational changes caused by the artificial intrasubunit disulfide bridge formation in the case of reoxidized large subunits or to conformational changes due to the disruption of disulfide bridges in the case of reduced large subunits.

By assaying the antifungal activity of two-by-two combinations of several antifungal proteins (chitinase, β-1,3-glucanase, *Urtica dioica* agglutinin, α-PT, *Amaranthus caudatus* antimicrobial proteins, *Mirabilis jalapa* antimicrobial proteins, *R. sativus* antifungal proteins, radish 2S albumins) we observed strongest synergism with the α-PT/radish 2S albumin combination (Terraș et al., 1992b). Although some synergisms between these antifungal proteins are known (chitinase with β-1,3-glucanase: Mauch et al., 1988; Leah et al., 1991; chitinase with the *U. dioica* agglutinin: Broekaert et al., 1989), some of these were not seen or were weakly displayed in our tests, which were initially performed on *B. cinerea* and *Colletotrichum lindemuthianum*. This could be due to different assay conditions and/or the use of fungi not showing a high sensitivity toward the above combinations of antifungal compounds. The results of a thorough analysis of the synergistic combination of antifungal thionins and 2S albumins are presented in this paper.

A clear enhancement of the α-PT antifungal activity was observed when 2S albumins, separate small and large subunits from the radish 2S albumins or 2S albumin-like trypsin inhibitors, were added at sub- or noninhibitory concentrations to a 2-fold dilution series of α-PT. The thionin $IC_{50}$ is lowered 2- to 33-fold upon addition of the different albumin preparations to half-strength PDB to a final concentration of 10 μg/mL. Under conditions where the α-PT antifungal activity is decreased and the albumin activity is completely abolished (half-strength PDB with 1 mM $CaCl_2$ and 50 mM KCl), the α-PT $IC_{50}$ is lowered 2- to 73-fold when the two proteins are combined. However, albumins have to be added at higher concentrations (up to 250 μg/mL) to obtain this synergistic effect. Furthermore, there seems to be an albumin level above which the synergism factor does not significantly

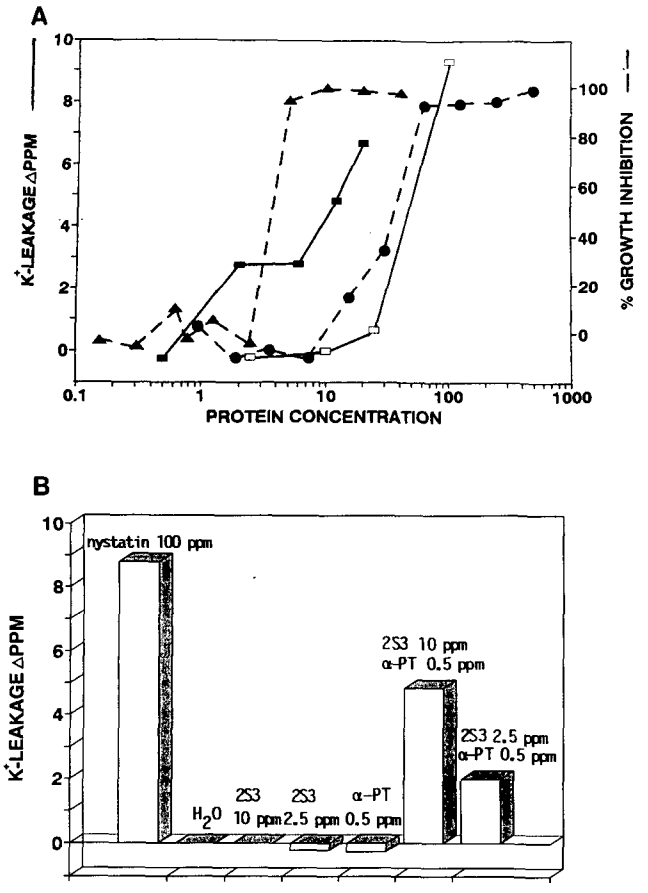


**Figure 3.** A, Correlation between $K^+$ leakage (measured by atomic absorption spectrometry 1.5 h after addition of the test protein to washed *F. culmorum* hyphae) caused by *Rs*-2S3 (□) and α-PT (■) and the percentage growth inhibition of *F. culmorum* (measured after 48 h of incubation at 25°C) by *Rs*-2S3 (●) and α-PT (▲). Protein concentration is in μg/mL. B, Illustration of the synergistic permeabilization ($K^+$ efflux measured 1.5 h after addition of test proteins to washed *F. culmorum* hyphae) of *F. culmorum* membranes by combinations of α-PT and *Rs*-2S3 (both at subpermeabilization concentrations) as determined by measurement of $K^+$ efflux. Nystatin and water were used as a positive control and a negative control, respectively.

increase. For the Brassicaceae 2S albumins, this level is about 50 μg/mL; for the *Hv*-Ti preparations, the level is 250 μg/mL or higher.

Thionins have a devastating effect on many, if not all, cell types. About 20 years ago, evidence was presented for the modification of the permeability of yeast membranes by these proteins (Okada and Yoshizumi, 1973). In the same report it was noted that divalent cations prevented the leakage of $K^+$ and UV-absorbing material. Later, it was found that thionins prevent growth of cultured mammalian cells by the same mode of action (Carrasco et al., 1981), and there is some evidence that the target site of thionins is a specific phospholipid domain in the membranes of these cells (Vernon and Rogers, 1992). By means of scanning EM and measurement of $K^+$ leakage, we showed that thionins also permeabilize hyphal membranes (preferentially close to the hyphal tips) and that at least the radish 2S albumins exert their antifungal activity by a comparable mechanism. Dose-response curves for hyphal growth inhibition by *Rs*-2S3 correlate with dose-response curves for membrane permeabilization (Fig. 3A), suggesting that membrane permeabilization is the major cause of growth inhibition by this protein. Although correlated as well, the dose-response curves for growth inhibition and membrane permeabilization by α-PT show a slight discrepancy in slope. This may indicate that growth inhibition by α-PT cannot be fully explained by membrane permeabilization alone or, alternatively, this discrepancy may be attributed to differences in test conditions for both assays. Furthermore, we showed that permeabilization of fungal membranes is greatly facilitated through interaction between thionins and 2S albumins (Fig. 3B).

It is also worth mentioning that thionins and 2S albumins display synergistic effects only on cell types that are affected by both types of proteins separately. For instance, most filamentous fungi and the bacterium *B. megaterium* are susceptible to growth inhibition by thionins as well as by 2S albumins and are synergistically inhibited by α-PT/2S albumin combinations. On the other hand, *S. lutea*, *E. coli*, yeast, human fibroblasts, and erythrocytes are sensitive to thionins but not to 2S albumins. Consequently, 2S albumins do not potentiate the toxicity of thionins on these cell types.

The question may also be raised whether the observed synergistic antifungal activity between thionins and 2S albumins has any biological relevance. In seeds of Brassicaceae, only one example of a thionin-like protein has been found, namely crambin from *Crambe abyssinica* (Garcia-Olmedo et al., 1989). This protein is not cytotoxic to any cell type and thus it is unlikely that a synergistic interaction occurs with the abundant 2S albumins. Our attempts to purify a thionin-like antifungal protein from radish seeds using a method similar to that described for the isolation of cereal thionins have been unsuccessful. However, wheat and barley grains contain both cytotoxic thionins and 2S albumin-like proteins with trypsin-inhibiting activities that are present at high amounts in the same seed tissue, namely the starchy endosperm (Bohlmann and Apel, 1991; Odani et al., 1983, respectively). It has also been demonstrated that thionin expression in barley leaves is induced after inoculation with a fungal pathogen (Bohlmann and Apel, 1991), whereas a Bowman-Birk trypsin inhibitor-like protein has recently been found to be systemically induced upon wounding of maize seedlings (Eckelkamp et al., 1993).

Recently, Molina et al. (1993) demonstrated that wheat thionins can also act synergistically in inhibiting bacterial growth with nonspecific lipid transfer protein-like proteins isolated from barley leaves. Nonspecific lipid transfer proteins are known to exert antifungal properties as well (Terras et al., 1992; Molina et al., 1993) and most probably interact with fungal membranes. However, it is not known whether nonspecific lipid transfer proteins affect membranes in a fashion similar to 2S albumins and whether the synergy between thionins and nonspecific lipid transfer proteins can also be explained by increased membrane permeabilization.

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