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# Analysis of the toxicity of purothionins and hordothionins for plant pathogenic bacteria

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**Abstract**

Purothionins (PTHs) and hordothionins (HTHs) were purified by cation-exchange chromatography from petroleum-ether extracts of wheat and barley flour respectively. The HTHs could be separated into two fractions, HTH-1 and HTH-2. Radial diffusion assays and micro-plate broth dilution assays with a number of plant pathogenic bacteria showed that these proteins were toxic for *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker on tomato, *C. m.* subsp. *sepedonicus*, the causal agent of ring rot on potato, and *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of a spot disease on tomato and pepper. Only minor differences in toxicity between PTHs and HTHs, and between HTH-1 and HTH-2, were detected. Minor differences in toxicity of these thionins were also detected for different strains of these bacteria. The use of these plant proteins for engineering bacterial disease resistance into solanaceous crops will be discussed.

*Additional keywords:* antibacterial, antimicrobial, genetic engineering, thionin, toxicity assay.

## Introduction

Bacterial diseases can cause a drastic decrease of yield in certain crops. Due to bacterial diseases, the losses of the worldwide potato production can be as high as twenty-five percent (Sawyer, 1984). Breeding for bacterial disease resistance therefore is of utmost necessity, but for most plant-bacterial pathogen combinations, traditional plant breeding has not been very succesful in this respect.

A different approach to control bacterial diseases in plants is offered by genetic engineering. The first genetically engineered plants which showed resistance to bacterial infection, were reported in 1989. Expression of a tabtoxin-resistant acetyltransferase in tobacco plants conferred resistance to *Pseudomonas syringae* pv. *tabaci* (Anzai et al., 1989). More recently, another example of this so-called 'pathogen-derived resistance' was reported. Expression of a toxin-resistant target enzyme in sensitive plants resulted in insensitivity towards the toxin and resistance to bacterial infection (De la Fuente-Martínez et al., 1992).

Another approach to engineer resistance in plants against bacteria, is the introduction and expression of proteins that have antibacterial properties. A family of genes of potential significance in this respect, are the genes coding for the so-called thionins (García-Olmedo et al., 1989; Florack et al., 1990). These are low-molecular-weight ($M_r \approx 5000$) plant proteins which almost all exhibit antimicrobial activity.

Thionins can be divided into five different types, based on the plant species and tissues in which they occur, the overall net-charge, the number of amino acids and disulphide bonds and the homology at the amino acid level (García-Olmedo et al., 1989; Castagnaro et al., 1992). The best studied are the highly basic type 1 thionins, which are abundantly present in the endosperm of most *Poaceae* (reviewed in García-Olmedo et al., 1989). These thionins all consist of 45 amino acids, eight of which are involved in four disulphide bonds. Thionins of this class share 85% homology at the amino acid level. Type 2 thionins have been identified in leaves of the parasitic plant *Pyrularia pubera* and in leaves of barley (*Hordeum vulgare*). These thionins consist of 47 or 46 amino acids respectively, are less basic and also have four disulphide bonds. Type 3 thionins have been identified in leaves and stems of a number of mistletoe species (*Viscum album*, *Phoradendron* spp. and *Dendrophtora clavata*) and consist of 46 amino acids with three disulphide bonds and are slightly basic. Type 4 thionins have been identified in seeds of the Abyssinian cabbage (*Crambe abyssinica*) and consist of 46 amino acids with three disulphide bonds and are neutral in charge. Type 5 thionins are also present in the endosperm of monocotyledons, but are neutral in charge, only consist of 36 amino acids and probably have two disulphide bonds (Castagnaro et al., 1992).

*In vitro* toxicity of the type 1 purothionins (PTHs) from wheat endosperm for a number of plant pathogenic bacteria (Fernandez de Caleya et al., 1972), and more recently, toxicity of the type 1 and 2 hordothionins (HTHs) from barley for two plant pathogenic fungi (Bohlmann et al., 1988) have been reported. The type 2 thionins might be directly involved in plant defence, since infection of barley with spores of powdery mildew, *Erysiphe graminis* f.sp. *hordei*, resulted in a transient increase of type 2-thionin mRNA levels in leaves (Bohlmann et al., 1988). In addition, cultivar-related differences in distribution of these type 2 thionins in epidermal cells were demonstrated in compatible and incompatible interactions between barley and powdery mildew (Ebrahim-Nesbat et al., 1989).

Our objective was to study the feasibility of using the type 1 HTHs for engineering bacterial disease resistance into a number of different plant species, especially into solanaceous crops. To this end, the toxicity of the HTHs for a number of bacterial pathogens causing serious damage on these plants, was determined first and compared with the toxicity of the PTHs, which were reported previously to inhibit growth of certain plant pathogenic bacteria (Fernandez de Caleya et al., 1972).

This study reports the purification of PTHs and HTHs from wheat and barley endosperm, and a comparative analysis of the *in vitro* toxicity of these purified thionins for a number of plant pathogenic bacteria. Possible applications of thionin encoding sequences for engineering bacterial disease resistance into crops will be outlined.

## Materials and methods

*Thionin extraction and purification.* HTHs were isolated from flour of seeds of *H. vulgare* cv. Femina, and PTHs from *Triticum aestivum* cv. Camp-Remy. Seed flour was extracted with petroleum-ether according to Békés (1975) and after concentration treated with hydrochloric acid in ethanol as described by Balls et al. (1942). The 'crude thionin' fraction was treated with water and ethanol as described by Fisher et al. (1968), to generate partially purified thionins. These were applied onto a CM Sepharose Fast Flow (Pharmacia) column and eluted with a linear gradient of 0.3 or 0.4 to 1.0 M NaCl in 50 mM phosphate buffer. Thionin containing fractions were pooled, concentrated and desalted by overnight dialysis against distilled water using Spectra/Por 6 (Spectrum) dialysis membranes (molecular weight cut-off 2000). The amount of protein was

estimated by the method of Bradford (1976). Before assaying toxicity, purified thionins were sterilized by filtration through Optex filters (0.22 μm; Millipore).

*Electrophoresis and Western analysis.* The molecular weight of the proteins present in the pooled fractions was determined by SDS-polyacrylamide gel electrophoresis on 12.5% gels in the presence of 7 M urea (SDSureaPAGE), as described by Swank and Munkres (1971). Molecular weight markers were from Sigma (MWS-877P) and a synthetic cecropinB ($M_r$ = 3832), added as an additional marker, was from American Peptide Company, Inc. (Sunnyvale, USA). The purity of the thionins was determined by electrophoresis of the samples over a 20% acidic-polyacrylamide gel (acidPAGE). The separating gel buffer contained 0.375 M HAc and 0.09 M KOH, pH 4.3. A stacking gel consisted of 4% polyacrylamide in 0.063 M HAc and 0.09 M KOH, pH 6.8. The single electrode buffer contained 0.035 M β-alanine and 0.017 M HAc. Samples were prepared in 0.017 M HAc, incubated at 70 °C for 10 min and allowed to cool to room temperature. Prior to loading, 1/20 vol loading buffer containing 0.063 M HAc, 0.09 M KOH, 1 M sucrose and 8.25 mM Pyronin Y (Sigma) was added to each sample. Gels were run at 180 V and 60 mA for 90 min on a 2050 Midget (LKB) electrophoresis unit. Gels were stained with Coomassie Brilliant Blue R-250.

Western analysis was performed according to Towbin et al. (1979) using an antiserum raised against the HTHs from barley endosperm (kindly provided by K. Apel, ETH Zentrum, Zürich, Switzerland) and an alkaline phosphatase-labelled secondary antibody.

*Bacterial strains and growth media.* Toxicity of thionins was tested for the following bacteria: *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker on tomato; *C. m.* subsp. *sepedonicus*, the causal agent of ring rot on potato; *Erwinia amylovora*, the causal agent of bacterial blight on a number of plant species; *E. carotovora* subsp. *atroseptica*, the causal agent of black leg on potato; *E. c.* subsp. *carotovora* and *E. chrysanthemi*, the causal agents of soft rot diseases on potato; *E. salicis*, the causal agent of the watermark disease on willow; *Pseudomonas solanacearum*, the causal agent of bacterial wilt on a number of solanaceous crops; *P. syringae* pathovar (pv.) *tabaci*, the causal agent of wildfire disease on tobacco; *P. s.* pv. *tomato*, the causal agent of a spot disease on tomato and *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of a leaf spot disease on tomato and pepper. The strains of these plant pathogenic bacteria used in this work, and the host plants from which they were isolated are listed in Table 1, and were kindly provided by the Netherlands Plant Protection Service, Wageningen, the Netherlands.

*Erwinia* spp. and *Pseudomonas* spp. were grown on bouillon-agar (BA) slants containing per liter: 8 g Lab Lemco broth (Oxoid), 5 g NaCl and 15 g agar (Oxoid) at 27 °C for 24 h. *C. m.* subsp. *michiganensis* and *X. c.* pv. *vesicatoria* were grown on growth-factor-agar (GFA) slants containing per liter: 0.4 g $KH_2PO_4$, 0.05 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.5 g $NH_4H_2PO_4$, 1 g glucose, 3 g yeast extract (Oxoid) and 15 g agar (Oxoid) (final pH 7.2) at 27 °C for 24 h. *C. m.* subsp. *sepedonicus* was grown on yeast-peptone-glucose-agar (YPGA) slants containing per liter 5 g yeast extract (Oxoid), 10 g peptone (Oxoid), 5 g D(+)-glucose and 15 g agar (Oxoid) at 20 °C for 72 h. The medium used for radial diffusion assays and for counting the number of CFU was trypticase-soy-agar (TSA, Becton Dickinson). Media used for micro-plate broth dilution assays were trypticase-soy-broth (TSB, Becton Dickinson) and a nutrient-broth (NB) described by Fernandez de Caleya et al. (1972), containing per liter: 0.1% beef extract (Difco), 0.2% yeast extract (Oxoid), 0.5% peptone (Oxoid) and 0.5% NaCl (final pH 6.5).

*Toxicity assays.* Inocula were prepared by resuspending bacteria from 24 to 48 h old agar slants in sterile distilled water and visual adjustment to approximately $10^8$ CFU/ml using MacFarland turbidity standards (McFarland, 1907). The actual number of CFU was determined by pour plating 100 µl of serial ten-fold dilutions in 300 µl TSA in 24-well Cluster[24] (Mark II, Costar) tissue culture plates.

Toxicity of thionins was measured by two methods. For the radial diffusion assay, 9 cm Petri dishes, containing a bacterial suspension of approximately $10^6$ or $10^7$ CFU in a total volume of 15 ml TSA, were used. Fifteen µl solutions containing 1, 5, 25 or 125 µg thionin in water were applied to 4 mm wells, cut in the agar plate using a sterile cork borer. Fifteen µl solutions containing 25 µg bovine-serum-albumin (BSA) and 15 µl water were used as controls. Toxicity of thionins was measured by screening for the presence of growth inhibition zones around the wells after incubation at 27 °C for 24 to 72 h, except for plates containing *C. m.* subsp. *sepedonicus* which were incubated at 20 °C for 72 to 96 h.

In the micro-plate broth dilution assay thionins were tested in serial, two-fold dilutions in flat-bottom 96-well microtiter-plates (Greiner). The final concentrations of thionins tested were 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128 and 256 µg/ml in TSB or NB. To each well 200 ml medium was added, containing the thionin and approximately $10^6$ CFU/ml of the bacterium to be tested. Plates were incubated without shaking at 27 °C for 24 to 48 h, except plates containing *C. m.* subsp. *sepedonicus* which were incubated at 20 °C for 72 to 96 h. After shaking of the plates for a few seconds on a micro-plate shaker, growth was measured turbidimetrically at 620 nm using an Easy Reader EAR400 AT micro-plate reader (SLT Lab. Instruments). CFU were determined as described above for the control well, for the last well with measurable bacterial growth, and for each of the wells in which no growth was observed.

## Results

*Purification of thionins.* The method described here resulted in approximately 100 mg freeze-dried crude HTH and 50 mg crude PTH from 2200 g barley flour and 1200 g wheat flour, respectively. Column chromatography of the crude HTH preparation (Fig. 1A) resulted in a number of fractions under two distinct peaks, which were positive for thionins, as estimated by Western analysis (Fig. 2B). These were collected in two separate pools, referred to as HTH-1 (fractions no. 18 to 26) and HTH-2 (fractions no. 27 to 33). Fractions under a third peak were negative for thionins. Column chromatography of crude PTH (Fig. 1B) resulted in a similar elution profile in which the fractions no. 16 to 25 appeared to be positive for thionins. These fractions were combined in a single pool and will be referred to as PTH. Concentration of pooled fractions, followed by desalting and lyophilization yielded 12.5 mg HTH-1, 34.5 mg HTH-2 and 21 mg PTH.

CM Sepharose column fractionation produced essentially pure thionin preparations (Fig. 2). The molecular weight of the pooled fractions was approximately 5000 (Fig. 2A), and Western analysis confirmed that these proteins were in fact thionins (Fig. 2B). The presence of bands of molecular weight 10 000, 15 000 and higher, might have been caused by intermolecular disulphide bonds between thionin molecules (multimeric forms), known to occur for PTHs due to their high cystein content (Redman and Elton, 1969). The purity of the preparations was confirmed by acidPAGE (Fig. 2C). Only a single band was visible in each of the preparations.

*Toxicity assays.* A fast and easy screening for toxicity of thionins was performed on strains of nine different plant pathogenic bacteria by the radial diffusion assay. Small

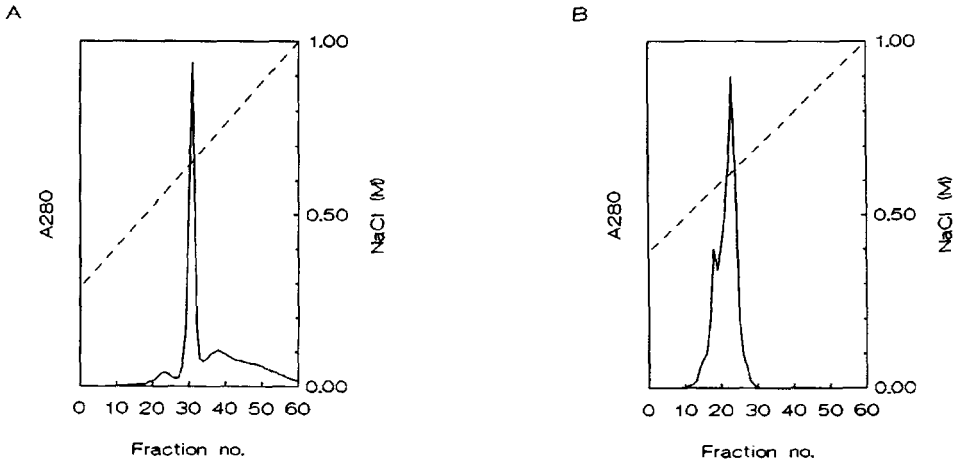


Fig. 1. Elution profiles of CM Sepharose Fast Flow column of crude hordothionins (HTHs) from barley (A) and purothionins (PTHs) from wheat endosperm (B). Symbols: —— , $A_{280}$; – – –, NaCl gradient. (A) Fractions 18 to 26 and 27 to 33 were pooled to render HTH-1 and HTH-2, respectively. (B) Fractions 16 to 25 were pooled to render PTH.

samples of HTH and PTH were applied to wells in bacteria-containing medium and bacterial sensitivity was measured by the presence of a growth inhibition zone around the well. *Erwinia* spp. and *Pseudomonas* spp. did not show growth inhibition zones for concentrations up to 125 μg HTH and PTH per well. However, *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus* and *X. c.* pv. *vesicatoria* exhibited growth inhibition zones in the presence of low concentrations of HTH or PTH (Table 1).

The diameter of the growth inhibition zones increased in a dose-dependent manner and was independent of the number of bacteria plated, $10^6$ or $10^7$ CFU per plate, or the thionin pool tested, HTH or PTH (data not shown). Two different strains of each of these three sensitive species, and of *E. amylovora* as a negative control, showed comparable results. A radial diffusion assay showed that the fractions 34 to 60, which were positive for the presence of protein but negative for HTHs, did not contain antibacterial activity when tested with up to 125 μg of protein per well against *X. c.* pv. *vesicatoria* (data not shown). To quantitate the antibacterial activity of PTH and HTH, bacteria were grown in broth in the presence of different concentrations of thionins and growth was measured turbidimetrically and by CFU counting. All bacteria in the control wells grew to densities of approximately $10^9$ to $10^{10}$ CFU/ml within the period of testing. As expected, only the two strains of *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus* and *X. c.* pv. *vesicatoria* were sensitive, whereas none of the other species showed growth inhibition (Table 1). Toxicity levels of PTH, HTH, HTH-1 and HTH-2 for the sensitive species appeared to be very similar, only minor differences could be detected (Table 2).

These micro-plate assays indicated that thionins exhibit bactericidal rather than bacteriostatic activity for *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus* and *X. c.* pv. *vesicatoria*, as the $OD_{620}$ dropped below the OD at the start of the experiment and no colonies were formed after plating on solid medium. A nutrient broth (NB) was used instead of trypticase-soy-broth (TSB), to be able to compare the results of our toxicity assays with previously published data on the toxicity of PTHs (Fernandez de Caleya et al., 1972). For all the bacterial species tested, the results using either medium were closely similar (data not shown).

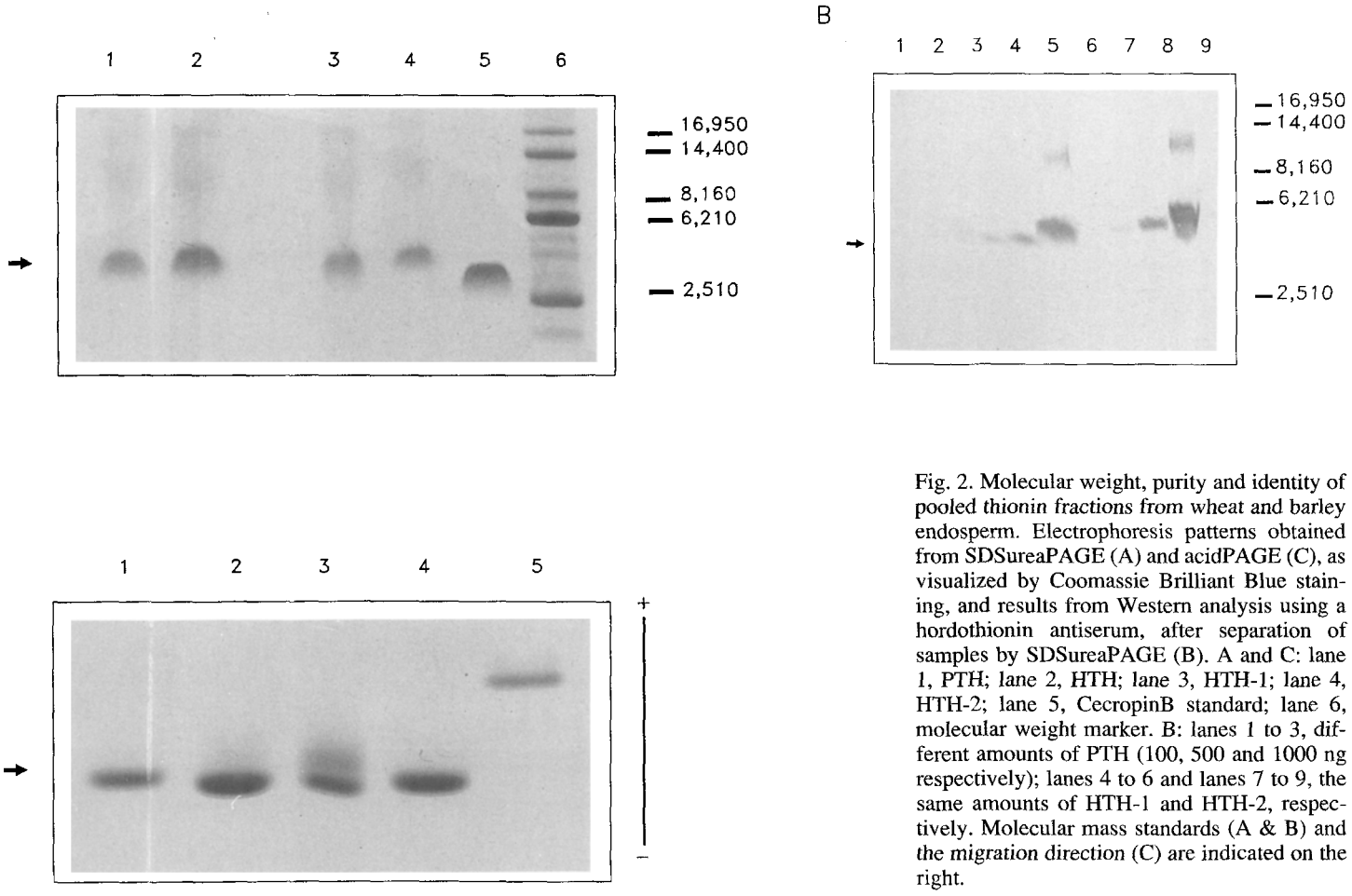


Fig. 2. Molecular weight, purity and identity of pooled thionin fractions from wheat and barley endosperm. Electrophoresis patterns obtained from SDSureaPAGE (A) and acidPAGE (C), as visualized by Coomassie Brilliant Blue staining, and results from Western analysis using a hordothionin antiserum, after separation of samples by SDSureaPAGE (B). A and C: lane 1, PTH; lane 2, HTH; lane 3, HTH-1; lane 4, HTH-2; lane 5, CecropinB standard; lane 6, molecular weight marker. B: lanes 1 to 3, different amounts of PTH (100, 500 and 1000 ng respectively); lanes 4 to 6 and lanes 7 to 9, the same amounts of HTH-1 and HTH-2, respectively. Molecular mass standards (A & B) and the migration direction (C) are indicated on the right.

Table 1. Bacteria tested in this work in radial diffusion assays (RDA) and micro-plate broth dilution assays (BDA) and results from these experiments.

| Microorganism | Strain[a] | Host[b] | RDA[c] | BDA[d] |
|---|---|---|---|---|
| *C. m.* subsp. *michiganensis* | PD520 (= NCPPB1468) | *Lycopersicon esculentum* | + | + |
| | PD1386 (=NCPPB1064) | *L. esculentum* | + | + |
| *C. m.* subsp. *sepedonicus* | PD1381 (=IPO270) | *Solanum tuberosum* | + | + |
| | PD37 (=IPO498) | *S. tuberosum* | + | + |
| *E. amylovora* | PD269 (=IPO108) | *Craetagus* sp. | – | – |
| | PD1387 (=IPO295) | *Pyrus* sp. | – | – |
| *E. c.* subsp. *atroseptica* | PD230 (=IPO161) | *S. tuberosum* | – | – |
| | PD1385 (=IPO281) | *S. tuberosum* | | – |
| *E. c.* subsp. *carotovora* | PD877 (=IPO857) | *S. tuberosum* | | – |
| | PD878 (=IPO858) | *S. tuberosum* | | – |
| *E. chrysanthemi* | PD226 (=IPO502) | *S. tuberosum* | – | – |
| | PD483 (=IPO871) | *S. tuberosum* | | – |
| *E. salicis* | PD1382 (=Dsk147) | *Salix* sp. | – | – |
| | PD1383 (=Dsk152) | *Salix* sp. | | – |
| *P. solanacearum* | PD134 (=IPO674) | *S. tuberosum* | – | – |
| | PD445 (=IPO933) | *S. tuberosum* | | – |
| *P. s.* pv. *tabaci* | PD1616 (=NCPPB1408) | *Nicotiana tabacum* | | – |
| | PD1617 =NCPPB1427) | *N. tabacum* | | – |
| *P. s.* pv. *tomato* | PD170 (=NCPPB1106) | *L. esculentum* | | – |
| | PD828 (=IPO920) | *L. esculentum* | – | – |
| *X. c.* pv. *vesicatoria* | PD1389 (=No.81-18) | *Capsicum annuum* | + | + |
| | PD1390 (=No.XV35) | *L. esculentum* | + | + |

[a] PD, collection Netherlands Plant Protection Service, Wageningen; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, UK; IPO, collection DLO Research Institute for Plant Protection, Wageningen; Dsk, collection DLO Institute for Forrest tree and Nature Research, Wageningen and No., collection University of Florida, IFAS, Bradenton, Florida, USA.
[b] Host plant from which the culture was isolated.
[c] RDA, radial diffusion assay; +, inhibition zone present and –, inhibition zone absent when tested with up to 125 mg PTH and HTH per well.
[d] BDA, micro-plate broth dilution assay; +, growth inhibition and –, no growth inhibition, as measured turbidimetrically at 620 nm when tested with up to 256 μg/ml PTH and HTH per well.

Table 2. Toxicity of thionins for sensitive plant pathogenic bacteria in μg/ml as determined by pour plating.

| Strain | | PTH | | HTH | | HTH-1 | | HTH-2 | |
|---|---|---|---|---|---|---|---|---|---|
| | | MIC[a] | MBC[b] | MIC | MBC | MIC | MBC | MIC | MBC |
| *C. michiganensis* subsp. *michiganensis* | PD520 | 64 | 128 | 32 | 64 | 64 | 64 | 32 | 64 |
| | PD1386 | 32 | 64 | 16 | 64 | 32 | 64 | 16 | 64 |
| *C. michiganensis* subsp. *sepedonicus* | PD37 | 16 | 32 | 16 | 32 | 16 | 32 | 16 | 32 |
| | PD1381 | 32 | 64 | 16 | 32 | 16 | 32 | 8 | 32 |
| *X. campestris* pv. *vesicatoria* | PD1389 | 16 | 16 | 16 | 16 | 16 | 16 | 8 | 16 |
| | PD1390 | 16 | 16 | 16 | 32 | 8 | 16 | 8 | 8 |

[a] MIC, minimal inhibitory concentration, the concentration at which growth was inhibited, as determined by CFU counting.
[b] MBC, minimal bactericidal concentration, the concentration at which more than 99.9% of the inoculum is no longer able to form colonies.

## Discussion

Our objective was to study the potential use of the type 1 HTHs for genetic engineering because these thionins are the best characterized at the molecular level. Nucleotide and amino acid sequences of HTHs have been published before, and hence could serve as an ideal starting point in the isolation and construction of HTH encoding sequences. However, the toxicity of the HTHs from barley endosperm for plant pathogenic bacteria was not tested before. To this end, these were isolated from barley endosperm, together with the PTHs from wheat endosperm for which toxicty data were available. Because of the ease and quality of the purification, type 1 thionins were recovered from the lipoprotein complexes present in petroleum-ether extracts. This yielded an almost pure and biologically active thionin preparation in a few steps.

The type 1 HTHs were previously shown to be a mixture of two highly homologous proteins, α- and β-HTH (Redman and Fisher, 1969). HTH-1 and HTH-2 may represent α- and β-HTH respectively, according to the elution from cation-exchange columns (Redman and Fisher, 1969). No differences in mobility on SDSureaPAGE and acidPAGE could be detected for all the thionin pools examined, probably due to the high overall homology in molecular weight and charge (reviewed in García-Olmedo et al., 1989) and the limited resolution of the gel system used.

The type 1 thionins from wheat endosperm were shown to be toxic for a number of bacteria (Stuart and Harris, 1942; Fernandez de Caleya et al., 1972), some of which are pathogenic for plants. The possible mode of action is absorption onto the cell membrane, causing a change in permeability by the formation of ion-channel pores that result in leakage, as was shown for yeasts (Okada and Yoshizumi, 1973) and for a mouse fibroblast cell line (Oka et al., 1992). These studies were performed with the PTHs. As a fast assay to determine the toxicity of HTHs, a radial diffusion assay was used. Our results demonstrated that by this method only three bacterial species of the nine tested were sensitive for the HTHs and PTHs. The sensitive bacteria were: *C. m.* subsp. *michiganensis*, the causal agent of bacterial canker on tomato; *C. m.* subsp. *sepedonicus*, the causal agent of ring rot on potato and *X. c.* pv. *vesicatoria*, the causal agent of a spot disease on tomato and pepper. The same results were obtained by testing in broth in micro-plates.

Apart from *C. m.* subsp. *michiganensis* (MIC 450 μg/ml, MBC 450 μg/ml), *C. m.* subsp. *sepedonicus* (MIC 1 μg/ml, MBC 1 μg/ml) and an undefined pathovar of *X. campestris* (MIC 56 μg/ml, MBC 110 μg/ml), also *E. amylovora* (MIC 540 μg/ml, MBC 540 μg/ml) and *P. solanacearum* (MIC 5 mg/ml, MBC 5 mg/ml) were reported to be sensitive at the indicated doses (Fernandez de Caleya et al., 1972). Our results with PTH, HTH, HTH-1 and HTH-2 showed a tenfold higher toxicity for *C. m.* subsp. *michiganensis*, but slightly lower toxicity for *C. m.* subsp. *sepedonicus* and surprisingly no toxicity for *P. solanacearum*. Toxicity of thionins for *E. amylovora* was not tested at the high doses indicated above. The reported difference of *P. solanacearum* in sensitivity for PTHs is in agreement with the results of others, who also did not find toxicity of PTHs against *P. solanacearum* when tested with up to 500 μg/ml PTH (Broekaert, personal communication). Differences might have been caused by differences in strain and race specificity. The strains we used, PD134 and PD445, were race 3 isolates, whereas the strain and race specificity used by Fernandez de Caleya et al. (1972) was not indicated. A direct comparison is further hampered by a number of experimental differences. The toxicity assays reported earlier were performed in a nutrient broth with crude PTHs isolated from an unknown cultivar of *T. aestivum*. Differences in toxicity between thionins isolated from different cultivars might occur and could explain the observed discrepancies. However, radial diffusion assays performed with crude PTHs isolated from another, unrelated wheat

cultivar, *T. aestivum* cv. Kraka, showed similar results (data not shown). In addition, the PTHs isolated from the wheat cultivars Manitou (Jones and Mak, 1976) and Manitoba No. 3 (Ohtani et al., 1975) were identical in amino acid sequence. Differences in toxicity resulting from the use of different growth media can be excluded because no significant differences, resulting from the use of TSB or NB, were observed.

Fernandez de Caleya et al. (1972) also investigated the difference in toxicity of α-PTH (a mixture of α1- and α2-PTH) and β-PTH, purified by ion-exchange chromatography according to Fisher et al. (1968), and found species-dependent differences for *P. solanacearum* and *X. c.* pv. *phaseoli*. We could not demonstrate such species-dependent differences in toxicity of the two HTH fractions for the sensitive bacterial species.

In conclusion, our results show that HTHs from barley and PTHs from wheat endosperm show comparable toxicity for *C. m.* subsp. *michiganensis*, *C. m.* subsp. *sepedonicus* and *X. c.* pv. *vesicatoria*, the causal agents of bacterial diseases in solanaceous crops. Minimal inhibitory concentrations (MIC) ranged from 16 to 64 μg/ml and the minimal bactericidal concentrations (MBC) were usually twice the MICs. Hence, both the PTHs (Fernandez de Caleya et al., 1972) and the HTHs (this study) are potent antibacterial proteins only needed in relatively low concentrations to kill plant pathogenic bacteria. The genes encoding the HTHs have good prospects for genetic engineering bacterial disease resistance in *Solanaceae*, which lack these proteins, which confirms the findings of García-Olmedo et al. (1989) for the PTHs.

We have made several gene constructs encoding different HTH precursor proteins to study the feasibility of engineering bacterial disease resistance into solanaceous crops. These have been introduced in tobacco, tomato and potato and we are now studying the expression and biological activity of the HTHs produced in these transgenic plants.

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