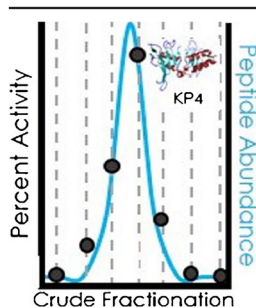


Fungal Secretome Analysis via PepSAVI-MS: Identification of the Bioactive Peptide KP4 from *Ustilago maydis*

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Abstract. Fungal secondary metabolites represent a rich and largely untapped source for bioactive molecules, including peptides with substantial structural diversity and pharmacological potential. As methods proceed to take a deep dive into fungal genomes, complimentary methods to identify bioactive components are required to keep pace with the expanding fungal repertoire. We developed PepSAVI-MS to expedite the search for natural product bioactive peptides and herein demonstrate proof-of-principle applicability of the pipeline for the discovery of bioactive peptides from fungal secretomes via identification of the antifungal killer toxin KP4 from *Ustilago maydis* P4. This work opens the door to investigating microbial secretomes with a new lens, and could have broad applications across human health, agriculture, and food safety.

Keywords: Bioactive peptides, Antimicrobial peptides (AMPs), Fungal secretomes, Peptidomics, Mass spectrometry, Natural products

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Introduction

Fungi represent a diverse and versatile kingdom that has survived on earth for over 500 million years [1]. While ~100,000 fungal species have been described to date, it is estimated that 5 million fungal species could exist [2]. This vast and largely unexplored kingdom represents a rich source of untapped natural compounds that have been crucial for their survival. Used for defense, fungi produce a wide variety of molecules to leverage competitive advantage over invading microbial pathogens. Fungi–microbe interactions have led to the production of antimicrobial compounds that can be harnessed for human health, agricultural, and food safety applications [3, 4]. Important among these are secreted small, cysteine rich antimicrobial peptides (AMPs).

Recent discoveries increasingly reveal bioactive peptides from a variety of sources to have substantial structural diversity and broad applications [5, 6]. The antimicrobial peptide, copsin, from the basidiomycete *Coprinospora cinerea*, was first discovered in 2014 and has potent activity against gram-positive bacteria, including both the human pathogen

Enterococcus faecium and the foodborne pathogen *Listeria monocytogenes* [7]. Additionally, there has been a growing interest in the agricultural use of bioactive peptides for crop protection, including transgenic expression in plants and topical application as biopesticides. Spear-T, a bioinsecticide derived from spider venom and marketed by Vestaron, has recently been approved by the EPA for commercial use. Importantly, unlike many currently used neonicotinoid-containing products, Spear-T has no adverse effects on bees or other beneficial insects, highlighting additional benefits of certain peptide-based agricultural products [8]. Fungi–microbe interactions have also inspired the use of these natural peptides in food safety applications, including the use of bacteriocins from lactic acid bacteria as starter cultures in food fermentation [9].

The promise of novel and effective mechanisms of action (MOAs) has revitalized peptide natural product discovery. In conjunction with methods aimed to expand knowledge of fungal genomes [10, 11], methods for rapid AMP identification of these species are required. Current methods employed for AMP identification include bioassay-guided approaches, which rely on multiple rounds of fractionation, require large amounts of material, and often bias toward highly abundant or highly active compounds. Alternative genome mining approaches leverage the advantage of economical deep

sequencing technologies but require knowledge of antimicrobial gene clusters or amino acid sequences a priori and offer no direct measure of bioactivity. As such, efficient and versatile methods are needed to screen potential AMPs against common and emerging pathogens. We developed PepSAVI-MS (Statistically guided bioactive peptides prioritized via mass spectrometry) to expedite discovery of bioactive peptides and validated this pipeline via identification and characterization of cycloviolacin O2 from the plant species *Viola odorata* (sweet violet) [12]. PepSAVI-MS utilizes selective extraction and fractionation of peptide source material, whole-cell bioactivity screening, and a statistics-guided mass spectrometry-based approach for targeted identification of putatively bioactive compounds. To expand the search for potent and effective AMPs, we now extend this pipeline to fungal secretomes, a rich source of AMPs with potentially novel MOAs created and refined by extreme inter- and intra-species competition [3, 7, 13, 14].

Herein, we demonstrate expansion of PepSAVI-MS to fungal-sourced AMPs using the killer toxin KP4 from *Ustilago maydis* P4 as proof-of-principle. KP4 is a highly positively charged 11.0 kDa peptide secreted by the corn smut fungus *U. maydis* when infected with the dsRNA P4 virus. The host and virus have co-evolved such that the host is not affected by the dsRNA virally-encoded toxin but when secreted by the host the toxin kills all other strains of *U. maydis*, thus giving the strain a selective advantage over other *U. maydis* strains [15, 16]. Two adaptations were required to demonstrate applicability of PepSAVI-MS for fungal secretome analysis: (1) an approach to harvest peptides secreted into the growth media replaced the extraction procedure, and (2) the bioassay format was adapted to screen against relevant fungal species. Successful application of PepSAVI-MS to microbial secretomes establishes the utility of this pipeline for novel fungal bioactive peptide discovery.

Experimental

Fungal Strains and Growth Conditions

Ustilago maydis P4 and P6 strains were acquired from Robert Bozarth at Indiana State University and Jeremy Bruenn at Buffalo State, respectively. All *U. maydis* strains were grown in complete *U. maydis* media (UM media) consisting of 2.5% peptone (BD Difco), 1% dextrose (Sigma Aldrich), 0.15% ammonium nitrate (Sigma Aldrich), and 0.1% yeast extract (Sigma Aldrich). Four 5 mL starter cultures of *U. maydis* P4 were added to 2 L UM media and were grown to late-log phase at 25 °C. Cells were removed by centrifugation (500 × g for 5 min) and the supernatant was collected.

Secretome Peptide Harvesting

The collected media was adjusted to a pH of 5.5 and stirred overnight with 140 mL of CM Sephadex C-25 resin (GE Healthcare) hydrated in 25 mM sodium acetate, pH 5.5. Slurry

mixture was gravity packed into a column and washed with two column volumes of 25 mM sodium acetate, pH 5.5, to remove unbound components. Peptides were eluted with 90 mL of 25 mM sodium acetate, pH 5.5, with 1 M NaCl, buffer exchanged into PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄), pH 7.3, using 3 kDa spin concentration filters (Millipore) and concentrated 10×.

Creation of Peptide Library via Crude SCX Fractionation

Four hundred twenty µL of the concentrated peptide secretome sample was subjected to a 40-min SCX method using a PolySulfoethyl A column (100 mm × 4.6 mm, 3 µm particles, PolyLC). A salt gradient was employed using a linear ramp of 5 mM ammonium formate, 20% acetonitrile, pH 2.7 to 500 mM ammonium formate, 20% acetonitrile, pH 3.0. Fractions were collected in 1-min increments and desalted with three washes of 1.3 mL deionized water using a vacuum concentrator.

Bioactivity Screening

The susceptible culture selected for *U. maydis* P4 killing assays was *U. maydis* P6 (UMP6), a related *Ustilago* strain infected with the P6 virus. While also producing a deadly toxin of its own, KP6, these cells are susceptible to KP4. UMP6 fungal challenge cultures were grown for 5 d in 5-mL aliquots of complete UM media at 25 °C with 240 rpm shaking. Soft agar was prepared fresh on the 5th d by the addition of 1.5% bacto agar (BD) to complete UM media and cooled in a water bath to 48 °C for 1 h. UMP6 cultures were added to the cooled agar at the ratio of 5 mL fungus/250 mL agar. UMP6-infused agar was poured into 100 × 15 mL Petri dishes and once solidified, wells were carved into each plate using 10 µL pipette tips (4 wells/plate). UMP4 fractions were added to each well (50 µL/fraction), in duplicate. After compound addition, plates remained at room temperature until visible growth inhibition was present (~5 d). Radial zones of clearance were measured around the point of application.

LC-MS/MS Analysis of Peptide Library

The *U. maydis* P4 peptide library was analyzed via a nano-LC-ESI-MS/MS platform composed of a Waters nanoAcquity UPLC coupled to an AB Sciex TripleTOF 5600 QTOF mass spectrometer. Peptide fractions were diluted to ~0.2 µg/µL and acidified to 0.1% formic acid. Five µL of each sample were injected onto a trap column (NanoAcquity UPLC 2G-W/M Trap 5 µm Symmetry C18, 180 µm × 20 mm: Waters) before transfer to the analytical C18 column (10k PSI, 100 Å, 1.8 µm, 75 µm × 250 mm: Waters). Peptide separation was carried out at a flow rate of 0.3 µL/min using a linear ramp of 5%–50 % mobile phase B (mobile phase A, 0.1% formic acid; mobile phase B, 0.1% formic acid in acetonitrile) over 30 min. The MS was operated in positive ion, high sensitivity mode with the MS

survey spectrum using a mass range of 350–1600 m/z in 250 ms and information dependent acquisition (IDA) of MS/MS data, 87 ms per scan. For IDA MS/MS experiments, the first 20 features above 150 counts threshold and having a charge state of +2 to +5 were fragmented using rolling collision energy $\pm 5\%$. Each MS/MS experiment put the precursor m/z on an 8-s dynamic exclusion list. Auto calibration was performed every eight samples (8 h) to assure high mass accuracy in both MS and MS/MS acquisition. The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE [17] partner repository with the dataset identifier PXD006931. De-isotoped peak lists for each fraction were generated using Progenesis Q1 for Proteomics software (Non-linear Dynamics, ver. 2.0). Automatic processing settings were used to align and peak pick ions across all runs. Identified features were quantified using AUC integration of survey scan data based on the summed intensity of each de-isotoped feature. Data was exported as “peptide ion data” with the default parameters from Progenesis at the “Identify Peptides” stage in the software.

Statistical Modeling

Areas of interest in the bioactivity profile must be selected for subsequent data reduction and modeling. The bioactivity region for *U. maydis* P4 versus *U. maydis* P6 was defined based on the observed bioactivity profile as fractions 27–32. Using the PepSAVI-MS software package (<https://cran.r-project.org/package=PepSAVImS>) [12], background ions were eliminated through retention time (14–45 min), mass (2000–14,000), and charge-state (2–10, inclusive) filters to reduce the data to potential compounds of interest. Retention time filters were selected to eliminate background ions, mass filters to select for the common mass range of bioactive peptides, and charge state filters to eliminate unwanted small molecules. Peak-picked data were binned and filtered using the previously established workflow-informed criteria [12]. Briefly, binning was performed with a 0.05 Da window of features with identical charge states and filtering required a maximum abundance inside the extended bioactivity area of interest (25–34) with <1% of that abundance outside of the chosen window. The extended bioactivity region was defined as the region of bioactivity ± 2 fractions on either side to account for the increased sensitivity of mass spectrometry (i.e., bioactive compounds may be present in those fractions at concentrations too low to detect bioactivity). All features required a minimum abundance of 1000. All m/z species meeting these filtering criteria were modeled using the elastic net estimator with a quadratic penalty parameter specification of 0.001 to determine the contribution of each species to the observed overall bioactivity profile. The resulting list contains candidate compounds ranked in order of when they entered the model, such that the highest ranked compounds have the greatest likelihood to be contributing to the bioactivity.

KP4 Purification

KP4 was purified as reported previously [18–20]. In brief, the toxin was isolated from the supernatant of the KP4 toxin expressing strain of *U. maydis* grown in complete *U. maydis* media for 7 to 10 d. Cells were removed by centrifugation at $10,000 \times g$ for 30 min. The supernatant was stirred overnight with CM Sephadex C-25 resin (Amersham Biosciences) equilibrated with 25 mM sodium acetate, pH 5.5. The toxin was eluted with 1 M NaCl using a Pharmacia GradiFrac system. The eluant was concentrated using a Minitan II Ultrafiltration System (Millipore) with 1 kDa cutoff membranes and dialyzed against a 10 mM malonic acid; pH 6.0. KP4 was then purified using a high-resolution cation-exchange chromatography (Mono-S; Amersham Biosciences) matrix attached to a fast-performance liquid chromatography system in the same buffer and using NaCl for elution. The toxin was further purified with size exclusion chromatography using an Amersham Biosciences Superdex-75 gel filtration column. Toxin activity was tested throughout the purification using killing-zone activity assays, and purity was assessed using Homogenous 20 SDS gels on an Amersham Biosciences Phastgel system. Only a single band representing KP4 was observable when silver staining was used to observe the protein bands.

KP4 Activity Validation

Purified KP4 was tested in the agar diffusion assay as described above. A dilution series of KP4 starting at 9 μM and decreasing 2-fold to 0.018 μM was used to determine the minimum inhibitory concentration. Erythromycin was used as a positive control at 100 $\mu\text{g/mL}$. Radii of inhibition were measured 4 d after application to UMP6-infused agar. Killing assays at all KP4 and erythromycin concentrations were performed in duplicate.

Results and Discussion

Overview

PepSAVI-MS implements a multipronged approach for bioactive peptide discovery that utilizes selective isolation and fractionation of peptides from source material, bioactivity screening, and mass spectrometry-based peptidomics for the identification of putative bioactive peptide targets. As PepSAVI-MS was originally demonstrated for expressed plant peptides, two minor adaptations were required to demonstrate applicability for fungal secretome analysis: (1) an approach to harvest peptides secreted into the growth media replaced the extraction procedure, and (2) the bioassay format was adapted to screen against relevant fungal species (Figure 1).

Creation of SCX Fractionated Libraries

Secreted peptides are collected from the cell-free supernatant using weak cation exchange resin added directly to the media. The peptide-resin slurry was gently washed to remove

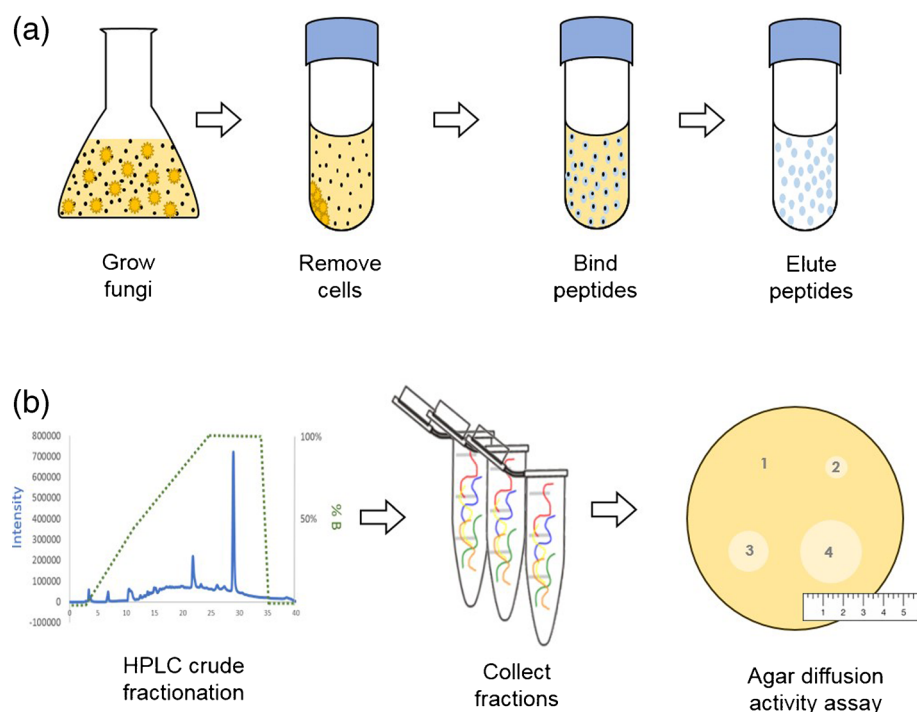


Figure 1. PepSAVI-MS pipeline modifications for secreted peptides. Modifications to the originally proposed PepSAVI-MS pipeline for **(a)** collection of secreted peptides, and **(b)** creation of fraction libraries and addition of each library to the adapted bioassay screen against relevant fungal species. Harvesting of secreted peptides includes large-culture microbial growth, secretome isolation via centrifugation, addition of weak cation exchange peptide binding resin to cell-free supernatant, and elution of peptides off of the resin. Eluted peptides are then fractionated into libraries that are subject to bioactivity screening via agar diffusion activity assays

unretained compounds, and then high salt was used to elute peptides/proteins. Then, SCX fractionation was used to create a UMP4 peptide library for bioactivity screening.

Bioactivity Screening

PepSAVI-MS is amenable to any developed bioassay and can be modified to accommodate any target pathogen. High-throughput 96-well microtiter-based assays for bacterial species were presented in the original demonstration of PepSAVI-MS [12]. However, this bioassay format is not amenable to fungal species that often fail to grow to high density and form fungal mats that interfere with accurate bioactivity measurements. For fungal species, traditional diffusion assays offer a tried-and-true format to obtain bioactivity data [21]. As such, agar-based diffusion assays were used to examine the activity of the *U. maydis* P4 SCX fraction library against a susceptible *U. maydis* P6 culture, showing strong activity across a discrete and well-defined region (fractions 27–32) (Figure 2a). The crude nature of SCX allows for a single peptide to elute across multiple sequential fractions and thus span a wide bioactivity region. This characteristic is crucial for modeling of bioactivity and LC-MS data, as more points across the curve allow for increased confidence in matched profiles. The elution profile of a select subset of peptides across SCX fractions (obtained via LC-MS analysis) has been plotted in Figure 3. On average, any given peptide elutes across 2–3 sequential fractions in varying abundances, and peptides are eluting across all regions of the

elution gradient. The combined elution and abundance profiles of each peptide create a unique fingerprint that allows for modeling with the bioactivity profile.

MS Profiling, Data Reduction, and Statistical Modeling

The *U. maydis* P4 SCX peptide library was subjected to LC-MS/MS analysis to obtain accurate mass and peptide abundance data for all constituents in the library. Peptide ion data reveals 17,473 features detected across the *U. maydis* P4 fraction set. Reduction of these features to those most likely contributing to the bioactivity in each region narrowed the number of features to 19. This large reduction in features (99%) is unusual for what we typically see with other peptide libraries (~96% reduction in features [12] and unpublished data). However, examination of fractions containing KP4 showed minimal peptidyl complexity in these particular fractions. Because KP4 is highly positively charged (10 basic residues), it retained on the SCX column much longer than other peptides and was detected in late eluting fractions. Statistical modeling using elastic net penalized linear regression of this filtered data set will still be useful to reveal a candidate list of compounds likely responsible for the bioactivity. When applied to the *U. maydis* data set, the four detected KP4 charge states (Figure 2b, c) meeting the filtering criteria ranked in the top 19 contributors [+7 (1st rank), +8 (5th), +9 (6th), +10 (4th)] to the bioactivity observed in fractions 27–32. Identification and ranking of multiple charge states for a given compound imparts

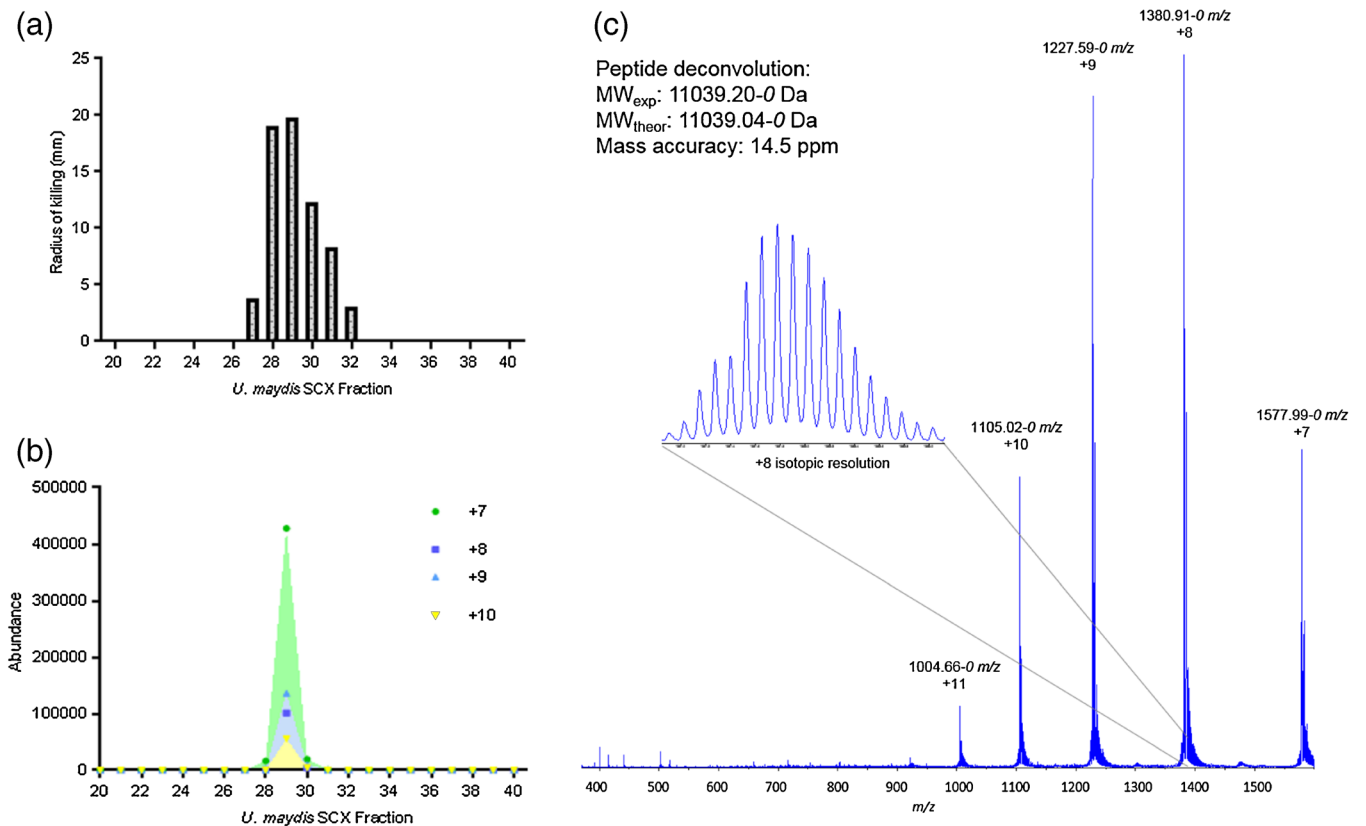


Figure 2. *U. maydis* bioactivity and KP4 LC-MS analysis. **(a)** *U. maydis* fraction library bioactivity screening against UMP6 with **(b)** aligned elution profile for KP4 as determined via manual charge state extraction. **(c)** Mass spectrum of intact KP4

a built-in compound redundancy that can help prioritize ranked species for downstream characterization and activity validation. The rank order of each charge state was independent of respective abundance, indicating PepSAVI-MS does not bias for

highly abundant species. While not done in this case, collapsing charge states via protein deconvolution prior to modeling would allow an opportunity for more unique compounds to rank highly.

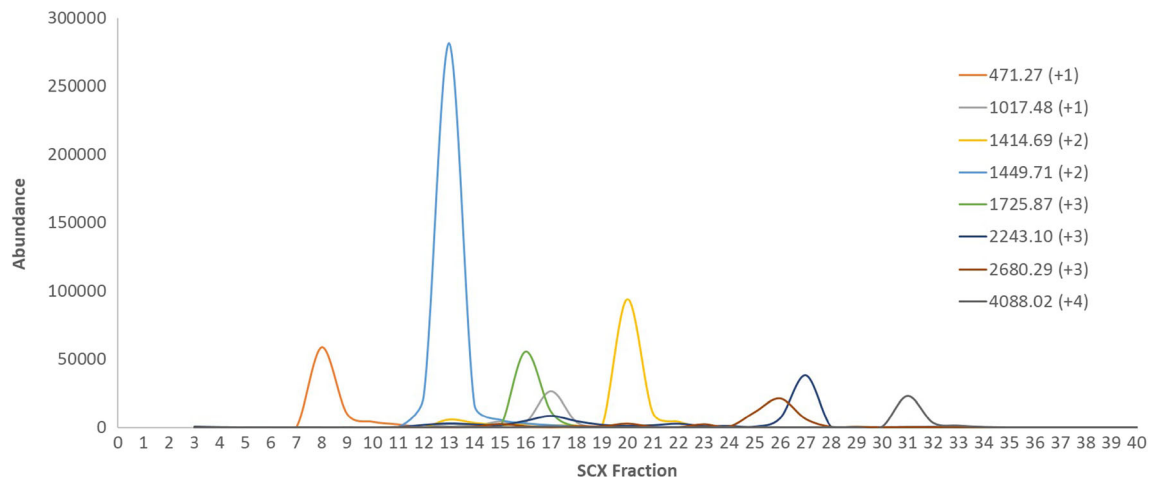


Figure 3. Peptide elution profiles across SCX fractions. Elution profiles for a select subset of peptides (denoted by their mass and detected charge state) generated from exported peptide ion data. Distributions are plotted for a range of peptides across SCX fractions with relatively similar abundances for observation on the same scale; however, lower and higher abundance species display similar elution profiles. Representative peptides elute across the entire SCX gradient with an average peak width of 2–3 fractions

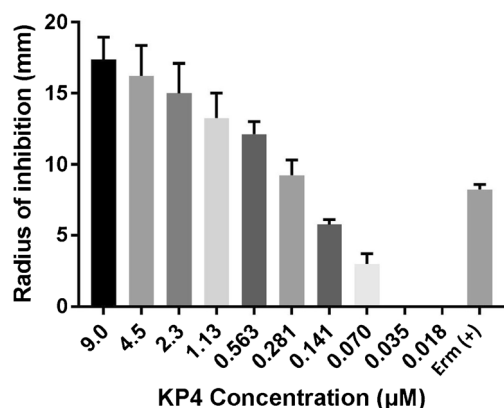


Figure 4. KP4 activity validation. Radii of inhibition observed through agar diffusion assays testing purified KP4 against the challenge culture *U. maydis* P6. KP4 was added to each well in varying concentrations using a dilution series of KP4 starting at 9 µM and decreasing 2-fold to 0.018 µM. Erythromycin [Erm (+)] was used as a positive control at 100 µg/mL. Zones of inhibition were measured 4 d after application to UMP6-infused agar. Killing assays at all KP4 and Erm concentrations were performed in duplicate

Activity Validation with Purified KP4

To confirm the activity of KP4, purified peptide was used in the agar diffusion-based assay against UMP6. KP4 was added to each well using a dilution series from 9 to 0.018 µM for minimum inhibitory concentration determination. After 4 d of incubation with KP6 infused agar, zones of inhibition were measured for each KP4 concentration. As seen in Figure 4, KP4 displays potent, reproducible antifungal activity in a dose-dependent manner at concentrations as low as 0.07 µM. No visible growth inhibition was observed at the two lowest KP4 concentrations (35 and 18 nM). Interestingly, these results suggest that KP4 is more potent towards *U. maydis* P6 cells than towards *U. maydis* P2 cells (MIC: 0.33 µM [22]). KP4 is also reported to inhibit *Arabidopsis* root growth (ED₅₀: 8 µM [23]) and expressed calcium channels (IC₅₀: 1.24 µM [16]) but, to our knowledge, this is the lowest reported inhibitory activity of KP4.

Successful application of PepSAVI-MS for fungal species demonstrates rapid and accurate identification of bioactive components from complex natural product secretomes and allows for focused downstream characterization and validation experiments on only the most promising species. Expansion of PepSAVI-MS to this historically rich source of bioactive molecules greatly enhances its capabilities and potential for novel bioactive peptide discovery. Furthermore, future implementations of PepSAVI-MS in combination with workflows that yield accessibility of new scaffold products such as genome mining of intractable fungal species [10] and strategies using advanced algorithms such as RiPPquest [24] or Pep2Path [25] leverage complementarity of each and continue to expand capabilities.

Conclusions

PepSAVI-MS is a versatile and easily adaptable pipeline for natural product bioactive peptide discovery. Minor modifications to peptide extraction/collection and bioactivity assay format facilitate exploration of fungal secretomes, a rich source of bioactive peptides. Successful application of PepSAVI-MS to microbial secretomes, as demonstrated with *U. maydis*, opens the door to investigating fungal species with a new lens and may have translational applications across human health, food safety, and agriculture.

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