Extraction and purification of ustiloxin A from rice false smut balls by a combination of macroporous resin and high-speed countercurrent chromatography

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Abstract

Rice false smut is an emerging plant disease worldwide. Ustiloxin A (UstA) is the major mycotoxin found in rice false smut balls, which are fungal colonies in rice florets. In this study, a new method consisting of macroporous resin column chromatography and high-speed countercurrent chromatography (HSCCC) was developed for UstA separation. UstA was extracted by a 3.81% HCOOH solution and adsorbed by XAD-4 resin. UstA was then eluted by a 40% methanol solution supplemented with 0.1% trifluoroacetic acid (TFA). Further purification was achieved by HSCCC using a two-phase solvent system consisting of n-butanol/TFA/H$_2$O (1/0.05/1, v/v/v). Under the optimized conditions, 225 mg of UstA was obtained with a purity of 97.39% in a single run, with a final recovery of 65.2%. An inhibitory effect on seed germination of wheat and maize caused by UstA was observed in a preliminary phytotoxicity assay.

Keywords: Ustiloxin A , Rice false smut, Preparative purification, Macroporous resin, High-speed counter-current chromatography, Phytotoxicity assay

Introduction

Rice false smut is a plant disease caused by the fungal pathogen *Ustilaginoidea virens* Takahashi, which is now rapidly emerging in rice cultivation worldwide (Qiu et al. 2019). The most noticeable symptom of rice false smut is the false smut balls that form on rice florets as orange or green bulbs (Fan et al. 2020). In addition to the yield loss caused by rice false smut, *U. virens* produces secondary metabolites called mycotoxins. There are two main types of mycotoxins in rice false smut balls,

namely, ustiloxins (Koiso et al. 1994) and ustilaginoidins (Meng et al. 2015). Ustiloxin A (Fig. 1), the main derivative of ustiloxins, has shown phytotoxicity against seed germination (Koiso et al. 1992) as well as cytotoxic activity on bovine brain tubulin in vitro (Ludueña et al. 1994) and early life zebrafish in vivo (Hu et al. 2019). UstA is also capable of causing "Lupinosis"-like lesions in mice (Nakamura et al. 1994). Hu et al. (2020) reported that UstA is produced early in *U. virens* infection and affects transcription in rice, which would probably cause decreased production.

Ustiloxins were found in almost every rice crop infected by *U. virens* and some high ustilotoxin-producing strains were separated from the false smut ball (Lin et al. 2018). As described by Cheng et al. (2019), UstA was found in the surface water of paddy fields in Hubei, China, and significantly disrupted the cell cycling of *Tetrahymena thermophila*, which make the ustiloxin

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contamination an emerging threat to food safety. Both the toxicological study and the risk assessment of UstA require a significant amount of the purified substrate. However, efforts towards the total synthesis of ustiloxins have been hindered by the asymmetric synthetic steps, and no feasible synthetic route has been reported, except for ustiloxin D (Cao et al. 2002). Shan et al. (2013) reported the purification of UstA and UstB by macroporous resins, yet the final relative content of UstA and B was 2.52 and 1.47%, respectively, far from that of the pure substance. No purification methods of pure UstA from rice false smut balls have been previously reported. Therefore, the direct extraction and purification of UstA from rice false smut balls are worthy of further investigation.

Macroporous resin (MR) and anion-exchange resin (AER) have shown high adsorption capacity and practical applicability and have been widely used in natural product purification. There are many reports representing the successive purifications of diverse natural products by MRs from different sources, including polyketides (Chen et al. 2016; Kuang et al. 2013; Wang et al. 2019; Zhang et al. 2007), terpenes (Dorni et al. 2017) and alkaloids (Cao et al. 2018). Nevertheless, it is difficult to obtain pure compounds solely by MR column chromatography. High-speed countercurrent chromatography (HSCCC) is a unique type of chromatography that is based on liquid-liquid partitioning. HSCCC is particularly feasible for natural product purification due to its high flexibility and sample recovery. The combination of MR and HSCCC is certainly practical for scaled-up production of natural products.

The purpose of this investigation is to establish an efficient method for the preparative purification of UstA from rice false smut balls by the combined use of MR column chromatography and HSCCC. The extraction parameters for UstA were optimized. The adsorption and desorption conditions of MR column chromatography were evaluated. The HSCCC parameters, including the solvent system and flow rate, were also investigated. A preliminary survey was conducted on the inhibitory activity of UstA against seedlings of rice, wheat and maize.

Material and methods
Chemicals and adsorbents
The analytical standard of UstA was kindly provided by Dr. Tomowo Kobayashi from the Research Institute of Sankyo Co. (Tokyo, Japan). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Hesse, Germany). Deuteroxide was purchased from Cambridge Isotope Laboratories, Inc. (Cambridge, Massachusetts, USA). All other chemicals were of analytical grade.

MRs including XAD-2, XAD-4, XAD-7HP, XAD-16 and XAD-1180 N and AERs including FPA90RF and IRA67 were purchased from Sigma-Aldrich (Shanghai, China). Prior to the adsorption study, all the resins were soaked in ethanol for 24 h and then washed with deionized water until no residual ethanol was observed in the eluted water.

Rice false smut balls were collected from a rice field in Jiangyan, Jiangsu, China.

Apparatus
HPLC analysis was performed on an e2695 system coupled with a 2998 photodiode array detector (Waters, Massachusetts, USA). The quantification of UstA was accomplished on an Empower3® workstation.

An OptiChrome-300 PLUS HSCCC system (Countercurrent Technology, Jiangyin, Jiangsu, China) coupled with a UV detector was utilized in the separation procedure. The system was equipped with two polytetrafluoroethylene (PTFE) multilayer coils (210 m × 1.6 mm id) with a total capacity of 300 ml and a 30 ml sample loop.

The final UstA product was identified by high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) analysis on a Triple-TOF™ 5600 system (AB Sciex, Foster City, California, USA) and a DRX-600 spectrometer (Bruker, Darmstadt, Hesse, Germany), respectively.

HPLC analysis of UstA and calculation of the adsorption capacity and desorption ratio
UstA was detected by using a high-performance liquid chromatography (HPLC) method. UstA quantification by HPLC followed the protocol of a previous study (Miyazaki et al. 2009). Briefly, each 10 μl sample was injected onto an ACE C18 column (ODS, 4.6 × 150 mm, 5 μm, Phenomenex, Guangzhou, Guangdong, China), and the mobile phase, water-methanol-phosphoric acid (400:100:1, v/v/v), was pumped into the system at a flow rate of 0.5 ml/min. UstA was detected at a wavelength of 254 nm at 35 °C. A calibration curve for UstA was established using the authentic sample, and sample quantification was accomplished by the external standard method. All the experimental data are presented as the means ± SD of three replicates.

The adsorption capacity and desorption ratio for UstA were calculated based on the following equations:

$$Q_e = \frac{V_0(C_0 - C_e)}{m}$$

$$D_r = \frac{V_d \times C_d}{m \times Q_e}$$

where Q_e represents the adsorption capacity (μg/g resin) and D_r represents the desorption ratio (%). C_0, C_e and C_d are the UstA concentrations in the initial sample

solution, adsorption equilibrium solution and desorption solution, respectively (μg/ml). V_0 and V_d are the volumes of the sample solution and desorption solution (ml), respectively, while m is the weight of the resin (g, dry weight).

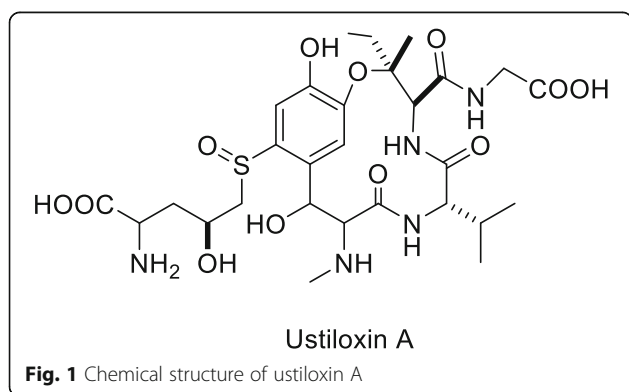## Extraction of UstA from rice false smut balls

Rice false smut balls were ground and sifted through a 30-mesh sieve before use. One hundred milliliters of each extraction solvent was added to a 250 ml Erlenmeyer flask preloaded with 10 g of false smut ball powder and then incubated at different temperatures with shaking at 180 rpm for 24 h. When optimizing the liquid-material (L-M) ratio, the amount of false smut ball powder was adjusted to the proper level.

Optimization of extraction parameters by response surface methodology

Response surface methodology (RSM) was utilized to optimize the extraction capacity of UstA. The optimal parameters were determined based on a Box-Behnken design. Three independent variables and optimized ranges were HCOOH concentration (X_1, 1, 3 and 5%), L-M ratio (X_2, 10:1, 15:1 and 20:1) and temperature (X_3, 25 °C, 31 °C and 37 °C), which were selected on the basis of preliminary experiments (Fig. 2). Seventeen runs with three replicates were generated and designed in Design-Expert 7.0 (Stat-Ease Inc., Minnesota, USA) (Table S1). The resulting degradation rates were fitted to the second-order polynomial equation as follows;

$$Y = b_0 + \sum b_i X_i + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2$$

where Y was the predicted response (in here the UstA concentration), b_0 was the constant, X_i and X_j were the variables, b_i was a coefficient, b_{ij} was the interaction coefficient and b_{ii} was the quadratic coefficient.

Fig. 1 Chemical structure of ustiloxin A

Separation of UstA by MR column chromatography

The extraction solvent containing UstA was adjusted by ddH$_2$O to a final concentration of approximately 300 μg/ml UstA. One gram of each pretreated resin was soaked in 50 ml of the sample solution and allowed to adsorb for 24 h with shaking at 180 rpm. Then, 500 μl of the sample solution was filtered and applied for HPLC analysis. The adsorption capacity was calculated by analyzing the residual UstA in the sample solution.

The effects of the sample solution pH on the adsorption capacity were investigated on two AERs. One gram of pretreated AER resin was soaked in 50 ml of the sample solution with three different pH values (3.0, 7.0 and 10.0), and static adsorption was allowed for 24 h. Then, the solution was sampled and applied for HPLC analysis.

The adsorption kinetics and equilibrium adsorption isotherms were studied by adding one gram of XAD-4 resin to 50 ml of the sample solution with different UstA concentrations and allowed to adsorb for 12 h at 25 °C. The residual UstA in the sample solution was then analyzed at the time intervals as described above.

The Langmuir and Freundlich models were used to represent the adsorption behavior between UstA and XAD-4 resin. The adsorption isotherms were calculated based on the following equations:

Langmuir equation:

$$Q_e = \frac{Q_m \times K_L \times C_e}{1 + K_L \times C_e}$$

where Q_e represents the adsorption capacity (μg/g resin) and C_e represents the UstA concentration in the adsorption equilibrium solution (μg/ml). K_L is the Langmuir adsorption equilibrium constant, and Q_m is the theoretical maximum adsorption capacity (μg/g resin).

Freundlich equation:

$$Q_e = K_F \times (C_e)^{\frac{1}{n}}$$

where Q_e represents the adsorption capacity (mg/g resin) and C_e represents the UstA concentration in the adsorption equilibrium solution (μg/ml). K_F is the Freundlich constant, while $1/n$ is an empirical constant.

Elution of UstA from the XAD-4 resin column

XAD-4 resin saturated with UstA was used to determine the elution solvent for UstA desorption. Each gram of saturated resin was put into a 50 ml Erlenmeyer flask and immersed in 20 ml of an aqueous methanol solution at ten different concentrations. All flasks were shaken at 180 rpm and room temperature for 6 h. The UstA content in the methanol solution was then analyzed by HPLC to determine the desorption capacity and desorption ratio.
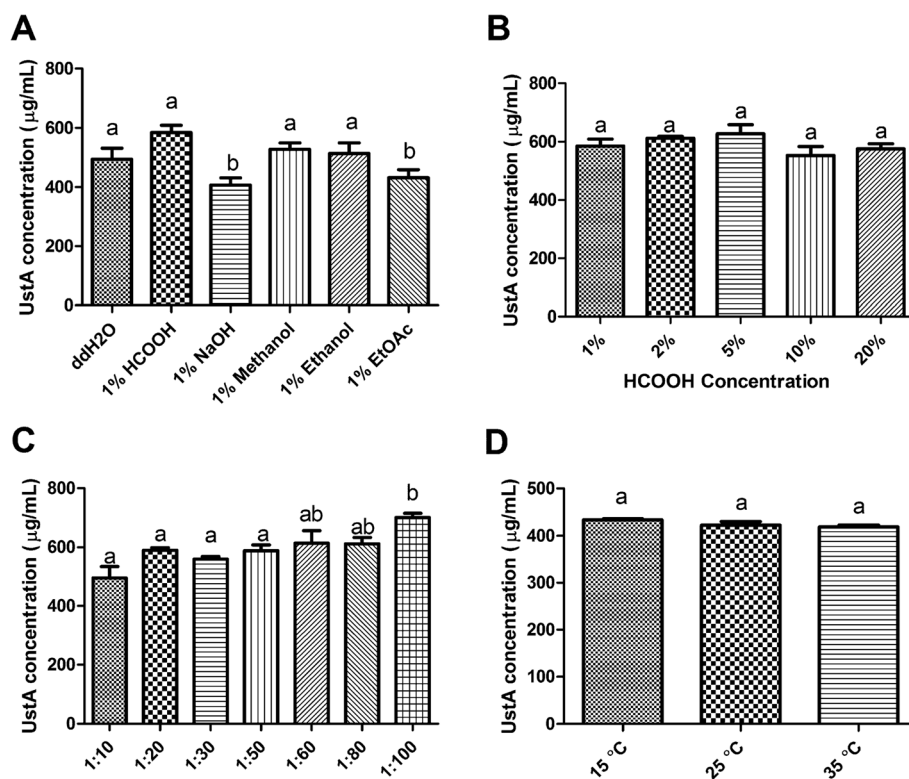
Fig. 2 The effects of different extraction conditions on the UstA extraction capacity. The extraction capacity was presented as the UstA concentration in the extraction solvent. **a** Different extraction solvents. **b** The effect of the HCOOH concentration on the extraction capacity. **c** The effect of the ratio of material and solvent on the extraction capacity; the UstA concentration was calibrated to a constant value by multiplying the dilution fold. **d** The effect of operating temperature on the extraction capacity. The values of columns with different letters were significantly different ($p < 0.05$)

The MR column saturated by UstA was used for dynamic desorption. The column was first washed with 1 BV of 5% methanol solution to remove water-soluble impurities. The eluting solvent (40% aqueous methanol) was then pumped into the MR column at a flow rate of 2.0 BV/h, and the eluting solution was fractionally collected and analyzed by HPLC. Trifluoroacetic acid (TFA) was added when necessary.

Selection of a two-phase solvent system for HSCCC separation

Samples eluted from the MR column were used for solvent system evaluation. Approximately 1 mg of the sample was added to a 10 ml screw-capped tube with 4 ml of the pre-equilibrated two-phase solvent system (2 ml of each phase). Then, the tube was shaken vigorously to mix the two phases and allowed to settle. The lower layer was filtered, and the upper layer (1 ml) was dried under a N_2 stream and dissolved in 1 ml of methanol; both layers were analyzed by HPLC. The partition coefficient (K value) of UstA was expressed as the ratio of the UstA content in the upper phase to that in the lower phase.

Structure elucidation of the UstA product

After the HSCCC system was equilibrated with the selected two-phase solvent system, samples pretreated with macroporous resin containing approximately 300 mg of UstA were dissolved in 20 ml of the mobile phase solvent and pumped into the HSCCC system at a flow rate of 1.0 ml/min. The operating temperature was set at 25 °C, and the revolution speed was 1000 rpm. The chromatographic spectra were recorded by a UV detector at 254 nm. The fractions corresponding to UstA were collected and evaporated under a N_2 stream to remove the solvent.

The obtained UstA product was dissolved in D_2O and aqueous methanol for NMR analysis as well as HRMS and HPLC analysis, respectively.

High-resolution mass spectrometry analysis was performed on an AB Sciex Triple-TOF™ 5600 system equipped with an ACE UltraCore SuperC$_{18}$ column (ODS, 3.0 × 150 mm, 2.5 μm, Phenomenex, Guangzhou, Guangdong, China). Gradient elution mode was used with the methanol/water system at a flow rate of 0.2 ml/min. ^{1}H- and ^{13}C-NMR spectra were measured in a D_2O solution on a Bruker DRX-600 spectrometer (600 MHz

for ^{1}H and 150 MHz for ^{13}C). The chemical shift (δ) was recorded in ppm relative to the solvent signals, and the coupling constant (J) was measured in Hz.

Inhibitory activity assay on the germination of plant seeds

Every 5 seeds of maize (Suke 5) or 10 seeds of rice (Nangeng 9108) and wheat (Yangmai 13) were inoculated into a 6-well plate precoated with 1 g of cotton. Next, 4 ml of deionized water or UstA solution was added to each well to make the concentration gradient of 200, 100, 50 and 20 μM. The plate was then incubated in the dark at 25 °C, and the length of each kernel was measured after 72 h. Glyphosate solution (1.7%) was used as a positive control.

Statistical analysis

At least three replicates were tested to calculate the mean value and the standard deviation. Quantitative data is expressed as mean ± SD and analyzed by ANOVA. Comparison between the groups was made by analyzing data with post-hoc method. Statistical significance was set at a level of $P < 0.05$. Multiple comparison between the groups was performed using Newman-Keuls multiple comparison test. Groups with significant difference were labeled with different letters.

Results

Optimized conditions for UstA extraction from rice false smut balls

Six kinds of solvents were tested in this study for the extraction of UstA from rice false smut balls, namely, deionized water, 1% HCOOH, 1% NaOH, 1% methanol, 1% ethanol and 1% EtOAc aqueous solution. As shown in Fig. 2a, the 1% HCOOH solution showed the best performance in UstA extraction and was chosen for further investigation.

Five HCOOH concentrations in the extraction solvent were set in this work. The UstA concentration in the extract solvent did not show significant differences at HCOOH concentration levels of 1, 2 and 5% and decreased when the HCOOH concentration was set at 10 and 20% (Fig. 2b). To avoid adverse effects on the subsequent isolation caused by the excessive acidity, the HCOOH concentration was set at 1% for UstA extraction.

We further optimized the ratio of the material and extraction solvent. In general, the total UstA amount increased with increasing ratio of material to solvent (Fig. 2c). However, a higher ratio resulted in too much extraction solvent and a lower UstA concentration in the extract. A ratio of 1:20 gave a superior extraction performance to that of 1:10; thus, the solid-liquid ratio was set at 1:20.

The effect of the extraction temperature was also investigated. As shown in Fig. 2d, no significant change was observed among the three different operating temperatures. Therefore, the extraction process was carried out at room temperature.

The optimal conditions of degradation and the interactive effects of pH, temperature and ZEN concentration were determined by RSM and the obtained results were fitted with a second-degree polynomial equation as follows,

$$Y = 640.26 + 19.27X_1 + 41.59X_2 - 2.26X_3 \\ + 1.65X_1X_2 - 17.15X_1X_3 \\ + 17.22X_2X_3 - 16.42X_1^2 + 10.56X_2^2 - 17.94X_3^2$$

where Y was the UstA concentration and X_1, X_2, X_3 were the coded independent variables.

The response coefficient (R^2) was 0.9509, which means that 95.09% of the response variability was covered by this model. The "Lack of Fit F-value" of 0.75 implies the Lack of Fit is not significant relative to the pure error ($P > 0.05$), indicating that the model was accurate enough to estimate the UstA extraction. Based on the regressive equation and the values of "Prob > F", we found that HCOOH content and L-M ratio and their interaction terms strongly impacted the UstA extraction performance, while operational temperature did not significantly affect the extraction ($P > 0.05$) (Table S2).

According to the analysis of Design-Expert software (Stat-Ease, Inc., Minnesota, USA), the optimal degradation conditions was at HCOOH content of 3.81%, L-M ratio 0f 19.97 and temperature at 31.87 °C when the maximal UstA concentration in the extract was calculated to be 698.51 μg/ml (Fig. S1).

Static adsorption comparison between the MRs and AERs

In the present study, five types of macroporous resin and two types of anion-exchange resin were investigated for their adsorption capacities, and their properties are stated in Table 1.

Figure 3a shows the adsorption capacities of the five macroporous resins (XAD-2, XAD-4, XAD-16, XAD-7HP and XAD-1180 N) for UstA. Although all the MRs except XAD-7HP were nonpolar, XAD-4 possessed the best adsorption capacity, reaching 240.72 μg/g resin for UstA.

Given that the UstA molecule possesses two carboxyl groups and one sulfonyl group and hence is capable of dissociating in aquatic solution, we applied anion-exchange resins for UstA adsorption. The weakly basic anion-exchange resin IRA 67 and strongly basic anion-exchange resin FPA90RF were tested for their adsorption capacity for UstA at different solution pH values. As shown in Fig. 3b, the adsorption capacities significantly increased when the extraction solution became

Table 1 Physical properties of macroporous and ion-exchange resins used in this study

Resins	Polarity	Mean pore size (Å)	Surface area (m²/g)	Pore volume (ml/g)	Matrix type
XAD-2	nonpolar	90	300	0.65	styrene-divinylbenzene
XAD-4	nonpolar	100	750	0.98	styrene-divinylbenzene
XAD-7HP	weakly polar	300–400	380	0.5	acrylic ester
XAD-16	nonpolar	200	800	1.82	styrene-divinylbenzene
XAD-1180 N	nonpolar	300–400	450	1.4	styrene-divinylbenzene
FPA90RF	strong base anion	ND	ND	0.8	acrylic ester
IRA67	weak base anion	ND	ND	1.6	acrylic ester

ND No data available

alkaline, and IRA67 was a better adsorbent than FPA90RF, reaching 230.42 μg/g resin for UstA. However, XAD-4 still showed a higher adsorption capacity than IRA67 and was chosen for UstA isolation.

Adsorption kinetics and isotherms of XAD-4 resin
The adsorption kinetic curve of UstA on XAD-4 resin is presented in Fig. 4a. The adsorption capacity increased rapidly in the first hour and then slowed. The adsorption reached equilibrium in 5 h. After 7 h, however, a slight increase in the adsorption capacity was observed. Taking both the time consumption and adsorption efficiency into consideration, the adsorption time was set at 5 h with static adsorption.

The adsorption isotherm is represented as the relationship between the concentration of the adsorbate and the adsorption capacity of the adsorbent per unit amount under equilibrium conditions, which indicates the physicochemical mechanism of the adsorption process. Two isotherm equations, the Langmuir and Freundlich models, are frequently used to fit the adsorption behavior (Foo and Hameed 2010; Li et al. 2020; Wang et al. 2017).

Equilibrium adsorption isotherms of UstA on XAD-4 were investigated with six different substrate concentrations at 25 °C. As shown in Fig. 4b, the adsorption capacity increased as the UstA concentration increased and reached a plateau of saturation at the UstA concentration of approximately 40 μg/ml.

The plots of C_e/Q_e versus C_e and ln (Q_e) versus ln (C_e) gave two linear regression models, which represent the Langmuir and Freundlich models, respectively. Table 2 shows the parameters for both models for UstA adsorption on XAD-4 at 25 °C. Based on the calculated correlation coefficients (R^2), the Freundlich equation (R^2 = 0.9841) fits the adsorption behavior better than the Langmuir equation (R^2 = 0.3921). It is notable that the Q_m and K_L values in the Langmuir adsorption model were both negative numbers, indicating that the adsorption of UstA did not follow a monolayer adsorption process.

Dynamic desorption of UstA by methanol solution
To determine the proper eluent for the elution of UstA, ten different concentrations of methanol solution were tested for their desorption ability. The results (Fig. 5a)
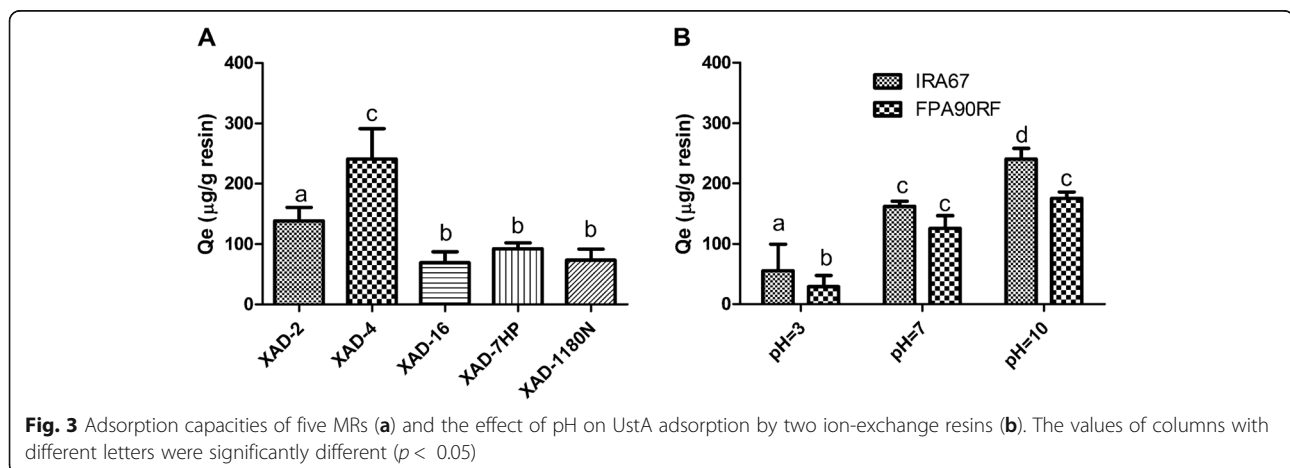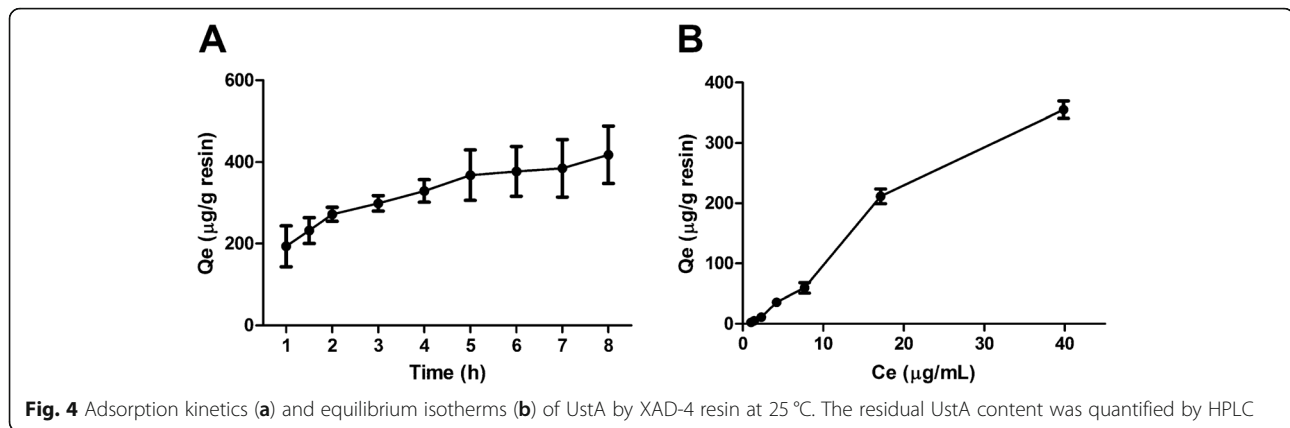

Fig. 3 Adsorption capacities of five MRs (**a**) and the effect of pH on UstA adsorption by two ion-exchange resins (**b**). The values of columns with different letters were significantly different ($p < 0.05$)

Fig. 4 Adsorption kinetics (**a**) and equilibrium isotherms (**b**) of UstA by XAD-4 resin at 25 ℃. The residual UstA content was quantified by HPLC

revealed that a higher methanol concentration led to a higher desorption capacity and ratio. When the concentration of methanol reached 40%, the desorption capacity remained stable. Therefore, 40% methanol solution was selected as the desorption solution due to the principles of efficiency and economy.

After the MR column was saturated, UstA was eluted by the 40% methanol solution at a flow rate of 2.0 BV/h. However, the elution curve for UstA exhibited severe tailing because UstA was dissociated into ions in the methanol solution. To overcome the peak tailing and accomplish the elution within a reasonable period, TFA was added to the eluent to a final concentration of 0.1%. As shown in Fig. 5b, TFA greatly enhanced the elution capacity of the eluent, and UstA was completely eluted with 3 BV. The recovery of UstA in this step was 82.6%.

Selection of the HSCCC two-phase solvent system for UstA purification

The separation ability of an HSCCC system depends on the distribution of substances in two immiscible liquid phases. Solvent system selection thus plays a vital role in successful separation by HSCCC. The partition coefficient (K value) is the most important parameter in solvent system selection because it determines the distribution of substances between the upper phase and lower phase solvents. It is suggested that an appropriate K should range from 0.5 to 2, while K exceeding the range would result in an inadequate resolution or prolonged elution. Several criteria have been developed for

the selection of solvent systems (Ito 2005). A total of eight solvent systems were investigated for UstA purification, ranging from low polarity to high polarity. The K values for UstA in each solvent system are recorded in Table 3. UstA is a highly polar compound and tends to dissociate into ions in aqueous solution; hence, UstA solely appeared in the lower phase, and the K value was zero in most solvent systems. The only promising system was the n-butanol/TFA/H_2O system, where UstA was distributed in both phases. When the TFA content was increased to 0.05, a proper K value of 0.62 was obtained. The resulting solvent system was used for the following separation of UstA.

It is noticeable that a stronger acidity of the solvent system would enhance the solubility and separation of UstA. However, in an HSCCC biphasic system, acidified solvent would cause several adverse effects, such as the emulsification and lower retention ratio of the stationary phase.

A preliminary survey was conducted at three flow rates, 1.0 ml/min, 2.0 ml/min and 4.0 ml/min. Although a higher flow rate reduces the elution time per run, it resulted in severe emulsification in the solvent system. The flow rate was therefore set at 1 ml/min.

Purification of UstA by HSCCC

After MR chromatography, the resulting fraction, which contained approximately 300 mg of UstA, was applied for HSCCC separation with the selected solvent system, that is, n-butanol/TFA/H_2O (1/0.05/1, v/

Table 2 Langmuir and Freundlich adsorption isotherm parameters for UstA on XAD-4 resin at 25 ℃ in the static experiment

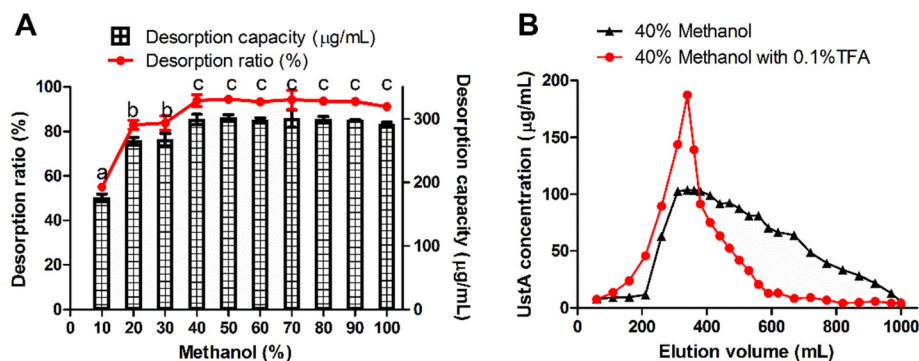| Langmuir adsorption isotherm		Freundlich adsorption isotherm	
Linear equation	$C_e/Q_e = -0.0065C_e + 0.2512$	Linear equation	$\ln(Q_e) = 1.4329\ln(C_e) + 1.1426$
Q_{max}	< 0	K_F	3.1349
K_L	< 0	$1/n$	1.4329
R^2	0.3921	R^2	0.9841

Fig. 5 Dynamic desorption for UstA. **a** Effect of methanol concentration on the desorption capacity and ratio. **b** Elution curve of UstA during the dynamic desorption process by using 40% methanol solution as the eluent. The values of columns with different letters were significantly different ($p < 0.05$)

v/v). Under the optimal HSCCC conditions, a baseline separation was achieved, and UstA was eluted from 160 min to 190 min (Fig. 6). The corresponding fractions were collected and combined to give a total of 225 mg of UstA in one single run.

The UstA content in the original extraction solution was 8.27%, as determined by HPLC analysis (Fig. 7a), while after MR chromatography, the relative content of UstA increased to 18.72% (Fig. 7b). The purity of the final UstA product was determined to be 97.39% (Fig. 7c), and the UV spectra of the product were in high accordance with those in the literature (Fig. 7d).

The UstA recovery in the HSCCC separation process was 78.9%, and the ultimate recovery of UstA was determined to be 65.2%.

Compound identification

The structure of the compound obtained by HSCCC was confirmed by ^{1}H-NMR and ^{13}C-NMR spectra (Fig.

S2), which are in high accordance with those recorded in the literature (Koiso et al. 1992).

Ustiloxin A: HR-MS: 674.2694 ([M + H]$^+$, $C_{28}H_{44}N_5O_{12}S^+$; calcd. 674.2707). ^{1}H-NMR (600 MHz, D_2O) δ_H 7.51 (s, 1H, H-13), 6.93 (s, 1H, H-16), 4.86 (d, $J = 10.0$ Hz, 1H, H-10), 4.81 (s, 1H, H-3, partially overlapped with the solvent peak), 4.32 (t, $J = 9.0$ Hz, 1H, H-3′), 4.24 (d, $J = 10.0$ Hz, 1H, H-9), 4.17 (d, $J = 4.5$ Hz, 1H, H-5′), 4.08 (t, $J = 10.0$ Hz, 1H, H-6), 3.92 (q, $J = 17.8$ Hz, 2H, H-19), 3.25 (dd, $J = 12.7, 3.0$ Hz, 1H, H-2′), 2.90 (d, $J = 12.7$ Hz, 1H, H-2′), 2.68 (s, 3H, 9-NCH$_3$), 2.19 (dd, $J = 13.7, 6.9$ Hz, 1H, H-22), 2.09 (m, 2H, H-4′), 1.80 (d, $J = 8.4$ Hz, 1H, H-24), 1.71 (s, 3H, H-21), 1.59 (dd, $J = 13.7, 6.9$ Hz, 1H, H-22), 1.00 (t, $J = 6.6$ Hz, 3H, H-23), 0.78 (d, $J = 6.1$ Hz, 3H, H-26), 0.66 (d, $J = 6.1$ Hz, 3H, H-25). ^{13}C-NMR (150 MHz, D_2O) δ_C 172.62 (C-20), 171.99 (C-6′), 170.61 (C-5), 170.30 (C-17), 165.25 (C-8), 151.80 (C-14), 145.53 (C-15), 135.78 (C-12), 127.21 (C-11), 123.60 (C-16),113.43 (C-13), 73.43 (C-10), 66.12 (C-9), 64.13

Table 3 The partition coefficients (*K* values) of UstA in different solvent systems

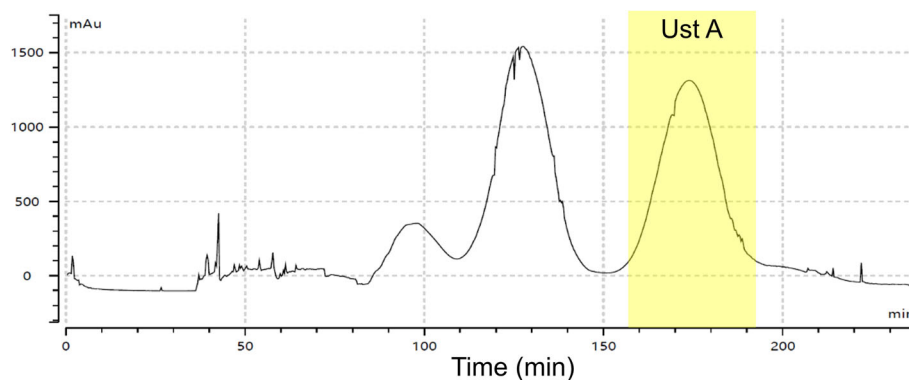| Solvent system	Ratio (v/v)	Settling time (s)	*K*-value
Chloroform/Methanol/n-Butanol/H$_2$O	4:3:0.2:2	17	0
Ethyl acetate/n-Butanol/H$_2$O	4:1:5	12	0
Ethyl acetate/n-Butanol/Methanol/H$_2$O	4:1:0.5:6	9	0
n-Heptane/n-Butanol/Methanol/H$_2$O	2:4:1:4	11	0
MTBE/Acetonitrile/H$_2$O	2:2:3	7	0
n-Butanol/Acetic Acid/H$_2$O	4:1:5	23	0.13
n-Butanol/TFA/H$_2$O	1:0.01:1	14	0.11
n-Butanol/TFA/H$_2$O	1:0.02:1	13	0.35
n-Butanol/TFA/H$_2$O	**1:0.05:1**	**14**	**0.62**

Fig. 6 HSCCC separation chromatogram of UstA. The solvent system was n-butanol/TFA/H$_2$O (1:0.05:1, v/v/v). Samples pretreated by MR containing approximately 300 mg of UstA were dissolved in 15 ml of the lower phase. The flow rate was 1.0 ml/min, and the chromatogram was recorded at 254 nm

(C-2′), 62.80 (C-3′), 59.52 (C-6), 58.80 (C-3), 50.79 (C-5′), 41.07 (C-19), 35.68 (C-4′), 31.58 (9-NCH$_3$), 28.12 (C-24), 20.78 (C-21), 17.86 (C-26), 17.59 (C-25), 7.23 (C-23).

Phytotoxicity of UstA on the germination of grain kernels

UstA has been reported to be phytotoxic and shows strong inhibitory activity against rice seedlings (Koiso et al. 1992). Herein, we tested the inhibitory effect of UstA on three kinds of grain kernels, namely, rice, wheat and maize. As shown in Fig. 8, the inhibitory activity of UstA on wheat and maize seedlings resembled that on rice, although the effects on wheat and maize were not as strong as those on rice. At the concentration of 20 μM, UstA significantly inhibited the germination of rice seeds; however, such a significant effect was observed above the concentration of 50 μM in the cases of wheat and maize seeds.

Discussion

Ustiloxins are acidic compounds due to the carboxyl and sulfonyl groups in the molecular structure, so an acidic solvent would improve the extraction performance. As in many cases, the acidic compounds were extracted with an acidic solvent (Beldean-Galea et al. 2015; da Rocha and Norena 2020; Kim and Kim 2015). Herein, we screened six water-based extraction solvents and selected the 1% HCOOH solution because it gave the best extraction capacity.

Ustiloxins tend to dissociate into anions in aqueous solution, so anion-exchange resins were tested for UstA adsorption (Bratkowska et al. 2012; Lee and Ku 1996). However, although the weakly basic AER IRA67 showed good adsorption capacity, XAD-4 MR exhibited better adsorption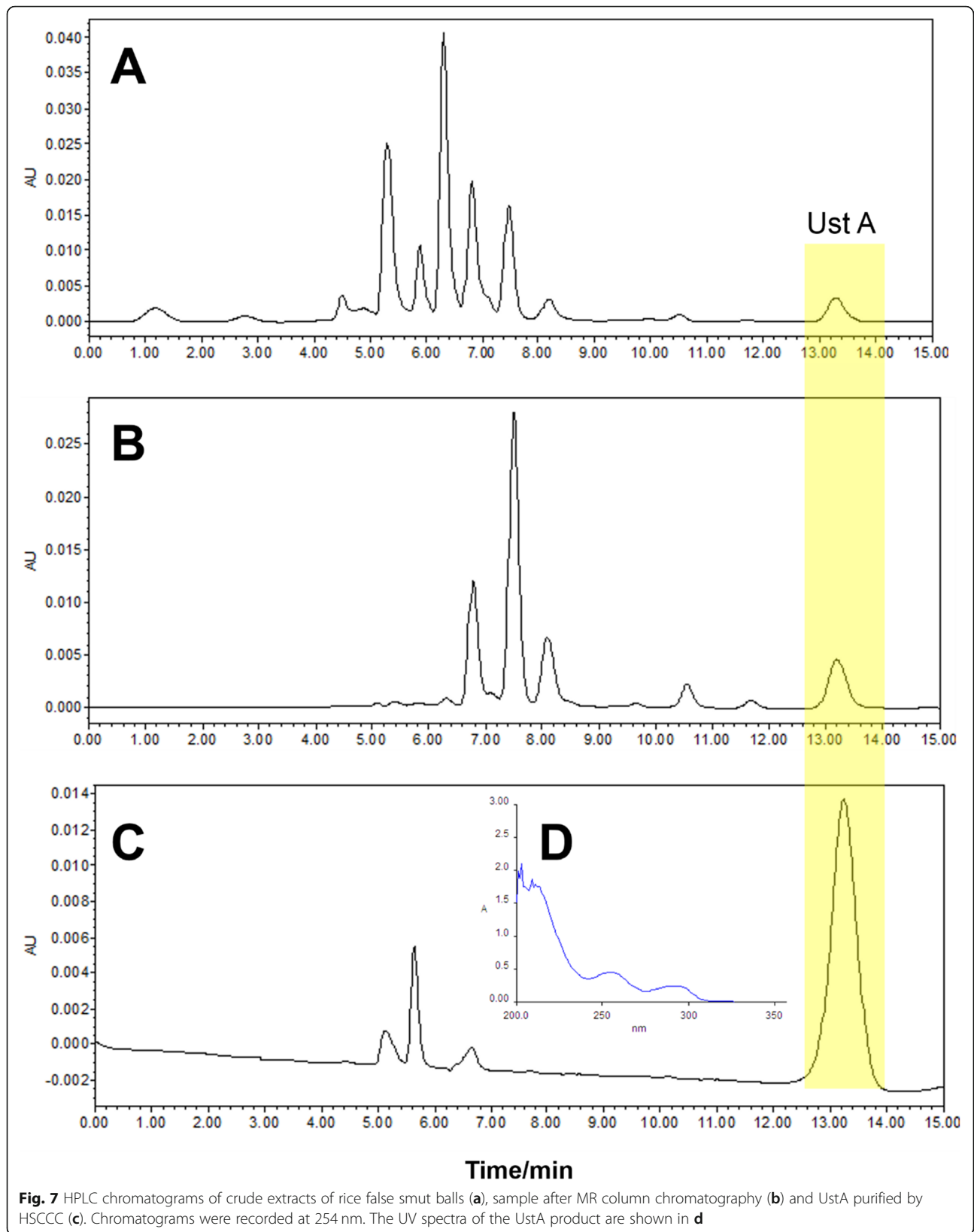 behavior. Furthermore, the application of MRs would greatly reduce the consumption of acid and alkali, which is more eco-friendly.

The isotherm of UstA on XAD-4 better fits the Freundlich model, which is an empirical model that accounts for repulsive interactions between adsorbed solute particles in addition to surface heterogeneities. The adsorption was unlikely to fit a monolayer model because the Langmuir model did not suitably fit the adsorption behavior.

When eluting UstA from XAD-4 resin by the 40% methanol solution, severe peak tailing occurred, resulting in a considerably prolonged elution time. TFA was added to the eluting solvent and greatly improved the elution capacity. We assumed that TFA inhibited the ionization of UstA and promoted the dissolution of UstA molecules in methanol solution, similar to the cases reported before (Arakawa et al. 2019; Tanaka et al. 1999).

Ustiloxins are highly polar compounds, which hindered our ability to develop a suitable solvent system for HSCCC separation. The n-butanol/H$_2$O system was selected due to its applicability in separating polar compounds, especially water-soluble substances. However, the retention ratio of the stationary phase in the n-butanol system is relatively lower than that in the low-polarity solvent systems. Moreover, this system is apt to emulsify at a high flow rate, limiting the separation efficiency.

The inhibitory effect of UstA on rice seedlings has been reported previously. Such effects were tested on the germination of wheat and maize seeds in this study, and the results showed that UstA also poses relatively low inhibitory activity on the seedlings of wheat and maize. As wheat and maize are frequently planted in the same field as rice in crop rotation, the phytotoxicity of UstA on wheat and maize should also be considered.
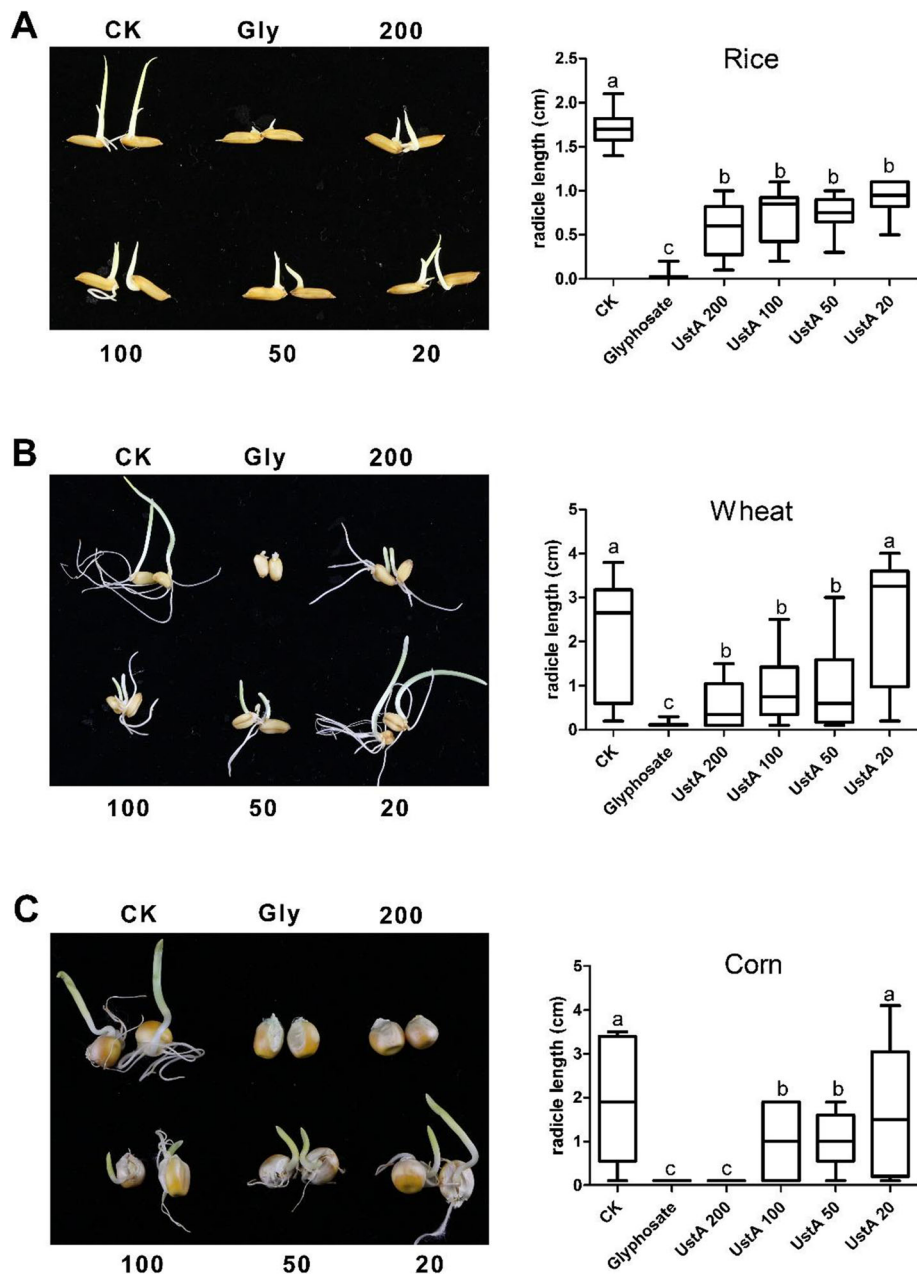
Fig. 7 HPLC chromatograms of crude extracts of rice false smut balls (**a**), sample after MR column chromatography (**b**) and UstA purified by HSCCC (**c**). Chromatograms were recorded at 254 nm. The UV spectra of the UstA product are shown in **d**

Fig. 8 Inhibitory activity of UstA on the radicle and germ elongation of the seeds of three different grains. **a** Rice variety Nangeng 9108, **b** Wheat variety Yangmai 13, and **c** Maize variety Suke 5. The concentration of UstA was represented in μM. The values of columns with different letters were significantly different ($p < 0.05$)

Conclusions

In summary, an efficient method based on MR column chromatography and HSCCC was established for the preparative separation and purification of UstA from rice false smut balls. Optimization for extraction, MR column chromatography and HSCCC conditions was carried out. Under the optimized conditions, 225 mg of UstA was obtained with a purity of 97.39% in a single

run. The ultimate recovery of UstA was 65.2%, confirming that this procedure is an effective method for mycotoxin production. A preliminary survey was conducted on the inhibitory activity of UstA on rice, wheat and maize seedlings. In addition to rice, we observed an inhibitory effect on the seed germination of wheat and maize caused by UstA.

Supplementary Information

> **Additional file 1.** Supplementary material associated with this article can be found in the online version.

Abbreviations
AER: Anion-exchange resin; HSCCC: High-speed counter-current chromatography; MR: Macroporous resin; TFA: Trifluoroacetic acid; Ust: Ustiloxin

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Not applicable.

Authors' contributions
GW and FZ conceived and designed research. GW, JH and DH conducted experiments. GW contributed to data analysis. GW wrote the manuscript. YWL, JS and JX reviewed and revised the manuscript. The authors read and approved the manuscript.

Availability of data and materials
All data in the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests.

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