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New production process of the antifungal chaetoglobosin A using cornstalks

Cheng Jiang[a], *Jinzhu Song*[a], *Junzheng Zhang*[b], *Qian Yang*[a,*]

[a] *Harbin Institute of Technology, School of Life Sciences and Technology, Harbin, Heilongjiang, People's Republic of China*
[b] *Harbin Institute of Technology, School of Chemical Engineering and Technology, Harbin, Heilongjiang, People's Republic of China*

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ABSTRACT

Chaetoglobosin A is an antibacterial compound produced by *Chaetomium globosum*, with potential application as a biopesticide and cancer treatment drug. The aim of this study was to evaluate the feasibility of utilizing cornstalks to produce chaetoglobosin A by *C. globosum* W7 in solid-batch fermentation and to determine an optimal method for purification of the products. The output of chaetoglobosin A from the cornstalks was 0.34 mg/g, and its content in the crude extract was 4.80%. Purification conditions were optimized to increase the content of chaetoglobosin A in the crude extract, including the extract solvent, temperature, and pH value. The optimum process conditions were found to be acetone as the extractant, under room temperature, and at a pH value of 13. Under these conditions, a production process of the antifungal chaetoglobosin A was established, and the content reached 19.17%. Through further verification, cornstalks could replace crops for the production of chaetoglobosin A using this new production process. Moreover, the purified products showed great inhibition against *Rhizoctonia solani*, with chaetoglobosin A confirmed as the main effective constituent (IC_{50} = 3.88 μg/mL). Collectively, these results demonstrate the feasibility of using cornstalks to synthesize chaetoglobosin A and that the production process established in this study was effective.

Introduction

Chaetoglobosins are a group of cytochalasins that exhibit strong cytotoxicity to various kinds of cells, including animal, plant, and microorganism cells. Chaetoglobosin A and B were the first members of this family discovered in 1973,[1] and since then over 40 analogs have been identified.[2] Chaetoglobosin A, which is biosynthesized mainly by *Chaetomium globosum*, is the most abundant member of this family[3] and displays various biological activities. Chaetoglobosin A shows highly toxic effects against human cancer cell lines, a murine leukemia cell line, and *Caenorhabditis elegans*,[3–5] and also shows phytotoxicity against alfalfa seedlings[6] as well as acute toxic effects against various types of microorganisms such as *Setosphaeria turcica*, *Rhizopus stolonifer*, and *Coniothyrium diplodiella*.[7–9] Despite these broad effects, development of an effective and economically feasible chaetoglobosin A production

* *Corresponding author*.
E-mails: microbio207@gmail.com, yangq@hit.edu.cn (Q. Yang).
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method remains challenging due to the low output by microorganisms and the high cost of the required culture substrates and purification process.

The only source of chaetoglobosin A is its biosynthesis by microorganisms. Therefore, several studies have been conducted with the goal of optimizing the type of culture substrates used to achieve higher yields, including oat, potato, malt extract, corn, molasses, rice, nut, and yeast extract-glucose.[10–14] The other important cost related to chaetoglobosin A production is the low initial content of the crude extract (below 5%), which increases the complexity and cost of production and purification.[8] Laboratory purification protocols usually rely on various combinations of thin-layer chromatography and Sephadex LH-20 columns to improve the product amount and content in samples before they are subjected to high-performance liquid chromatography (HPLC).[8,15] Although these steps may afford excellent results in terms of yield and purification, the current procedures for the recovery and purification of chaetoglobosins are still unsuitable for large-scale production.

A key step to developing a new economical production procedure is the choice of inexpensive substrates for the biosynthesis of chaetoglobosin A. *C. globosum* is a traditional antagonist that is widely used for biological control,[16] and has been confirmed to show excellent biodegradability of cellulose substrates[17] such as cornstalks. Cornstalk accounts for over 30% of the production of all cellulosic biomass in China, but is generally left in the fields after harvest or is even burnt[18]; thus, the vast availability of this resource shows its potential as a low-cost raw material for chaetoglobosin A production. Alternatively, use of a complex culture system is likely to introduce additional impurities, which could increase the difficulty and cost of purification. Therefore, this study was designed to demonstrate the feasibility of using cornstalks to replace crops as a culture substrate, which could reduce the cost of chaetoglobosin A production. Furthermore, the optimal conditions for obtaining a higher content of chaetoglobosin A in the crude extract were determined, including the optimal extractant, operation temperature, and pH value. After the above handling procedures, the biocontrol efficiency of the partly purified products against a pathogenic fungus was evaluated. Finally, the cost of the crude extract using cornstalks as a substrate was compared with that using crops as raw materials. Together, our study provides an effective method for the large-scale production of chaetoglobosin A with high content and effective antibacterial activity.

Materials and methods

Microorganisms and culture conditions

C. globosum W7 was obtained from the Microbial Genetic Engineering Lab of Harbin Institute of Technology and was found to have the capacity of chaetoglobosin A production in our previous study (unpublished data). The strain was preserved in the China General Microbiological Culture Collection Center under accession number CGMCC 3.14974. Fermentation was carried out in 250-mL flasks with 10 g of cornstalks, 0.5 g ammonium chloride, and 20 mL water by spreading 1-mL suspensions containing 10^7 spores/mL of *C. globosum*. The strain was incubated at 28 °C for 14 days and then the surface of the medium was completely covered by spores. The fungus and medium were taken out from the flasks, tiled on glass plates, and air-dried at 28 °C for 24 h. Before use, the cornstalks (Dahe Agriculture LLC., Tsitsihar, China) were first smashed into particles with a size less than 0.25 mm (60 mesh per inch) using a plant pulverizer (Beijing light medical equipment Co. Ltd., Beijing, China). The cornstalks used in this study contained 41.725 ± 0.148% (m/m) carbon and 0.805 ± 0.035% (m/m) nitrogen, according to the results of the elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). The medium consisted of cornstalks and ammonium chloride (nitrogen content 26.17%) with a ratio of 20/1 (w/w), so that the carbon/nitrogen ratio of the total medium was approximately 20/1.

Extraction effects of different solvents

The extraction effects of seven kinds of solvents were tested, including methanol, ethanol, ethyl acetate, acetone, dichloromethane, chloroform, and n-hexane. To reduce errors, ten flasks of fermentation residues were mixed and weighed after air-drying and smashing into particles with a size of less than 0.25 mm. Total smashed samples were averaged into 100 portions to ensure that each portion was fermented from about 1 g of cornstalk. The portions were then placed into 100-mL ground glass-stoppered flasks with the various organic solvents, respectively. Extraction was carried at room temperature (20–25 °C) for 24 h with 50 mL of organic solvent per flask, and this step was repeated twice.

After the extraction solvent and sample were separated with a G3 sintered glass funnel, the liquids were concentrated to about 2 mL using reduced pressure distillation (Shyarong Biochemical Instrument, Shanghai, China) under vacuum at 0.095 Pa, with final vacuum drying in Savant Speedvac (Thermal Technology LLC., Santa Rosa, CA, USA) at room temperature (20–25 °C). The distillation temperatures of different solvents were methanol at 50 °C, ethanol at 60 °C, ethyl acetate at 50 °C, acetone at 40 °C, dichloromethane at 40 °C, chloroform at 40 °C, and n-hexane at 60 °C. Dried extracts were redissolved in 1 mL acetone, and insoluble substances were removed via centrifugation at 15,000 × *g* for 10 min (Beckman Coulter Inc., Fullerton, CA, USA) before quantification of chaetoglobosin A with HPLC. After drying the solvent, the weights of the crude extracts were determined on a Precision Electronic balance (Sartious AG, Goettingen, Germany). All procedures were performed in triplicate independently.

Determination of optimal operating temperature

To determine the optimal temperature for chaetoglobosin A preparation, the crude extracts were dissolved into acetone with a final concentration of chaetoglobosin A of 1.0 mg/mL. Then, the Eppendorf tubes with 0.5 mL of the above solutions were placed at different temperatures (−20 °C, 0 °C, room temperature, 40 °C, 50 °C, 60 °C, 80 °C, 100 °C, 150 °C) for either 1 h or 24 h. Based on the results of the extraction effect test, acetone was chosen as the solvent. All procedures were

performed in triplicate, and then the remaining amount of chaetoglobosin A was quantified.

The influence of pH value on the crude extract

Crude extracts were quantified and then subpackaged to 0.5 mg chaetoglobosin A per sample. An equal volume of acid or base solution, prepared with hydrochloric acid or sodium hydroxide, respectively, under different concentrations, was added after the crude extracts were redissolved in 500 μL ethyl acetate, and the total liquids were mixed evenly for 1 h. The pH values of the hydrochloric acid were set as 0–5 and the pH values of sodium hydroxide were set at 9–14. Water (pH 7) and samples without dispose were set as the control groups. After the water solutions were removed and the solvents were dried, the weights of the remnant crude extracts and chaetoglobosin A were determined. All of the procedures were performed in triplicate.

Preliminary purification of the crude extract

n-Hexane, methanol–water, and sodium hydroxide solution were tested for optimization of the removal of impurities step. Based on the results of the extraction effect tests, the fermentation residues produced from 1 g of cornstalk were extracted with acetone and concentrated to 1 mL at 40 °C. Chaetoglobosin A was quantified by HPLC and all experimental samples were weighed after drying. All of the procedures were performed in triplicate. The group without any handling was set as the control.

Owing to its negligible effect for extracting chaetoglobosin A, n-hexane was used to degrease the lipids in the samples. The experimental sample was dissolved in 500 μL of methanol, and the same volume of n-hexane was added. After mixing for 1 h, the supernatant (n-hexane) was wiped off and the methanol was dried in vacuum. The amounts and contents of chaetoglobosin A were detected, respectively.

Sodium hydroxide solutions (0.1 M, pH = 13) were used for deacidification. The dried crude extract was redissolved in ethyl acetate, and an equal volume of lye was added. The remaining amounts of crude extract and chaetoglobosin A in the supernatants were detected after blending for 1 h.

Methanol–water was also applied for degreasing. Each sample was dissolved in 200 μL of methanol, and then 800 μL of water was added. After mixing for 1 h, insoluble substances were disposed by 15,000 × *g* centrifugation for 10 min, and the supernatants were freeze-dried.

Sodium hydroxide solutions and methanol-water were used in combination for purification. To prevent chaetoglobosin A from being destroyed by the organic acid in a water environment, the sodium hydroxide solution was first used for deacidification and the products from this step were considered as the first purified extract. Then, methanol–water was used for degreasing after the sample was vacuum dried at room temperature and the products from this step were considered as the second purified extract. As the last step, the treated extracts were dissolved in methanol after drying, and the final amounts and content of chaetoglobosin A were detected. All of procedures above were performed in triplicate.

Chaetoglobosin A detection

Detection of chaetoglobosin A in weighed samples was performed using an HPLC system equipped with an ultraviolet detector (Waters Corporation, Milford, MA, USA). An Agilent analytical column (TC-C18, 4.6 × 250 mm, 5 μm) was used to analyze the samples at room temperature under a flow rate of 1.0 mL/min. The mobile phase consisted of acetonitrile and water at a ratio of 45:55,[12] and the column temperature was 25 °C. The absorbance of samples was read at 227 nm[19] and the samples were analyzed using an Empower workstation (Waters Corporation, Milford, MA, USA). The chaetoglobosin A content in the samples was quantified in comparison with the standard substance (dissolved in methanol). The content of the crude extract was determined as the percentage of chaetoglobosin A relative to the total weight of the crude extract. All of the data were analyzed statistically using the software SPSS 19.0, and two-way analysis of variance was used to evaluate the significance of differences.

Gas chromatography–mass spectrometry (GC–MS) analysis of the crude extract

The compounds in the crude extract (dissolved in methanol) were analyzed to detect organic acids by GC–MS (Agilent 7890A-5975C, Santa Clara, CA, USA) according to a previously reported method[20] with minor modification. In this study, the compounds were separated with an Agilent column (HP-5MS, 30 m × 250 μm × 0.25 μm) and the GC temperature programs were as follows: initial temperature was 80 °C held for 2 min, increased to 180 °C at a rate of 5 °C/min, and then increased to 280 °C at a rate of 3 °C/min and held for 5 min, with a total run time of 60.33 min. The injector and detector temperatures were set at 280 °C. The ion source temperature was 230 °C, and the energy of the ionizing electron was 70 eV. Helium was used as the carrier gas at a flow rate of 1 mL/min. A 1-μL sample was injected in splitless mode with a solvent delay of 5 min. The characteristic ions and retention times of the target compounds were obtained and identified with full-scan mass spectra from *m/z* 50 to 500. The MS Spectrogram Database (http://www.organchem.csdb.cn/scdb/main/mss_introduce.asp) was used for data comparison.

Biocontrol efficiency of the second purified extract against Rhizoctonia solani

R. solani (CGMCC3.2888) was chosen to test the biocontrol efficiency of the prepared chaetoglobosin A on potato dextrose agar (PDA) plates (Φ9 cm). The second purified extract dissolved in ethanol was added prior to filtration for sterilization and introduced aseptically into the PDA medium (melting and cooling to about 60 °C) at different final concentrations (0, 0.5, 2.5, 5.0, 25, and 50 μg/mL). A slice (Φ0.8 cm) of *R. solani* mycelium was gently placed on the cooled agar and cultured at 28 °C for 120 h. PDA plates with the same volume of ethanol or no solvent were inoculated in the same manner and set as the control. The areas of mycelia were photographed and quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Bethesda, MD, USA) every 12 h for the sensitivity analysis of chaetoglobosin A. Finally, the calculated half-maximal

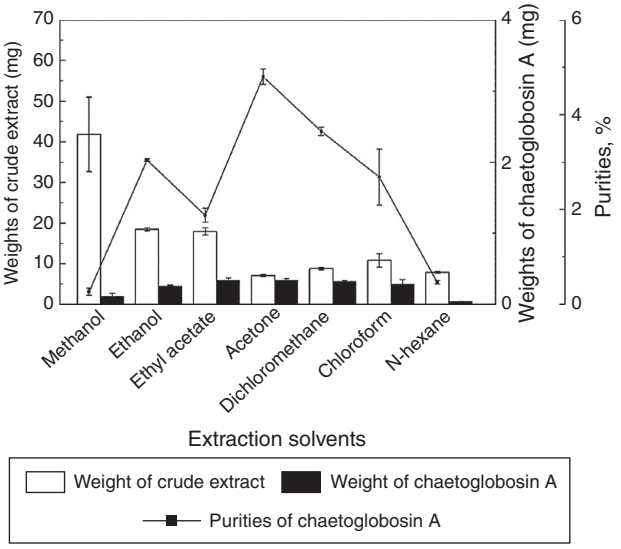

Fig. 1 – Effects of different solvents on the extraction of chaetoglobosin A. All extractions were performed on the fermentation residue of 1 g of cornstalk medium.

inhibitory concentration (IC_{50}) value (3.88 μg/mL) was verified and compared with that of the standard chaetoglobosin A (purity >98%, Sigma–Aldrich Co. LLC., St. Louis, MO, USA) at the same concentrations using the same experimental procedures. All of the tests were performed in triplicate.

Results and discussion

Extraction of chaetoglobosin A

Proper agents are vital for the extraction of chaetoglobosin A. According to previous studies, methanol,[21] ethyl acetate,[22] acetone, dichloromethane,[23] chloroform,[4] and n-hexane[24] were chosen to determine the best solvent for extraction of chaetoglobosin A. In addition, ethanol was also tested together with other solvents owing to its low cost and the fact that it is a primary alcohol (like methanol). Fig. 1 shows the total amounts of crude extract and the contents of chaetoglobosin A obtained when using different solvents for extraction.

As shown in Fig. 1, little chaetoglobosin A was extracted with n-hexane. However, there were still many substances present in the samples, indicating that this solvent might be useful for reducing impurities. Furthermore, the alcohols, especially methanol, could only leach a small amount of chaetoglobosin A, even though methanol had been commonly used as the extract solvent (only for mycelium) in previous studies.[21] These results suggested that chaetoglobosin A can be destroyed by some substances in the solid samples when alcohols were added to the fermentation products. The main candidate for this destruction was considered to be organic acids given that chaetoglobosin A was identified as an alkaloid based on its structure.[1] Approximate pH-value examination showed that the methanol-extracting solution is acidic (pH < 4) using extensive pH indicator paper. GC-MS analysis showed that the mass spectra of some compounds in the crude extract had high similarity with those in the database, and a portion of the compounds was organic acids, including acetic acid, butyric acid, succinic acid, p-hydroxy benzoic acid, and 3-indole-carboxylic acid. Overall, these results demonstrated that alcohols were not the most suitable as extract solvents for chaetoglobosin A in the fermentation residue of cornstalk medium. In addition, the abilities of the other four solvents to extract chaetoglobosin A were similar. However, samples with the highest contents were extracted when using acetone, which was thus chosen as the extraction agent for the sample preparation in subsequent experiments. Using acetone as the extractant, the output of chaetoglobosin A was 0.34 ± 0.02 mg/g cornstalk and the initial purification was 4.80 ± 0.16%. However, this content was not sufficient for application, considering the commercial concentrations of agricultural antibiotics (0.5–5%) based on the Database of China Registered Pesticides (http://cheman.chemnet.com/pesticides/). Therefore, to obtain a high-quality product with cornstalk, the crude extract needed to be purified.

Optimal handling temperature

Proper heat is required for the extraction and purification of chaetoglobosin A, but most antibiotics are labile at high temperature.[25] Hence, a comprehensive evaluation of the thermostability of chaetoglobosin A is indispensable to verify that the extract method in this study is feasible for its application. Hence, the samples of an equal concentration of chaetoglobosin A were placed at −20 °C, 0 °C, 40 °C, 50 °C, 60 °C, 80 °C, 100 °C, and 150 °C for 1 h or 24 h, respectively. The initial amount of chaetoglobosin A was 0.5 mg, and the control group was left at room temperature for the same amount of time. The difference in the content of chaetoglobosin A remaining was analyzed between each test group and control group. As shown in Fig. 2, chaetoglobosin A was scarcely reduced below room temperate (20–25 °C), showing that a temperate under 25 °C would hardly cause significant destruction. By contrast, a decrease in the amount of chaetoglobosin A was observed after heating at 60 °C, although only the treatment for 24 h showed a statistically significant level of reduction. When the samples were treated at 80 °C, 100 °C, and 150 °C for 1 h, chaetoglobosin A decreased significantly but did not disappear altogether. However, in the groups heated at these temperatures for 24 h, the chaetoglobosin A content sharply decreased, especially at 100 °C and 150 °C, in which no chaetoglobosin A was detected.

The thermostability of chaetoglobosin A determined in this study differs from that reported in a previous stud.[10] At the medium temperature tested (80 °C), the overall amounts of chaetoglobosin A remaining was the same as reported previously, whereas when the temperature exceeded 100 °C, the heat stability of chaetoglobosin A was improved with our method compared to that reported previously. Although other components present in the extract may also affect the thermal stability of chaetoglobosin A, the different heating method applied in this study influenced the results greatly. Other than the dried samples, the other samples used in this study were dissolved in solvents, which protected the chaetoglobosin A from being sharply destroyed. These results showed that it is

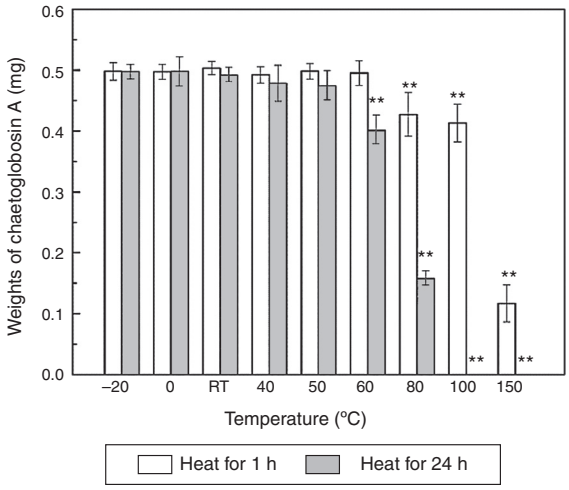

Fig. 2 – Thermostability of chaetoglobosin A placed at various temperatures for 1 h or 24 h. The initial amount of chaetoglobosin A in every sample was 0.5 mg. Asterisks indicate a statistically significant difference (*$p < 0.05$) in the amount of chaetoglobosin A remaining between the control temperature (room temperature, 20–25 °C) and the others. Two asterisks indicate a highly significant difference ($p < 0.01$).**

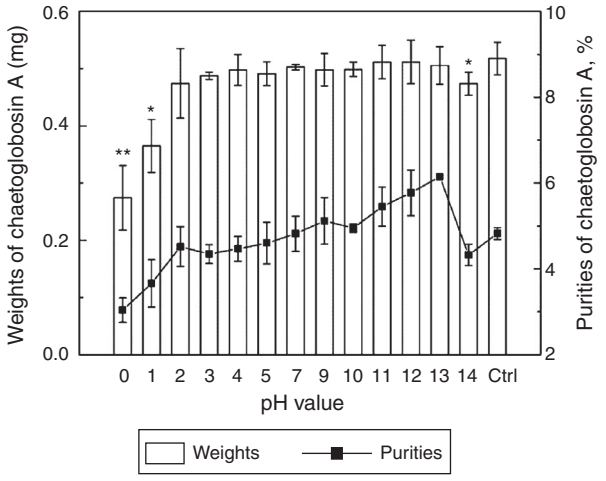

Fig. 3 – pH stability of chaetoglobosin A. The initial amount of chaetoglobosin A in each sample was 0.5 mg. Asterisks indicate a significant (*$p < 0.05$) or highly significant ($p < 0.01$) difference between the treatment groups and control.**

preferable for chaetoglobosin A to be dissolved in solvents and that the operation temperature should be maintained at less than 60 °C. Although a temperature below room temperature was found to be the most suitable, in consideration of reducing the drying time of the samples, 40 °C was chosen as the optimal processing temperature.

The effect of acid or alkali treatment on the crude extract

Before establishing the use of alkali in the purification procedure to remove the organic acids in the samples, the pH stability of chaetoglobosin A was verified with an acid and alkali. In a preliminary experiment, the samples were steeped in hydrochloric acid (1 M, pH = 0) or ammonium hydroxide (saturation, pH = 11.7). No chaetoglobosin A could be recovered after either treatment for 1 h, which indicated that direct contact with an acid or alkali was harmful to chaetoglobosin A. Therefore, ethyl acetate was used as the solvent to protect chaetoglobosin A in subsequent tests, owing to its insolubility in water.

As shown in Fig. 3, chaetoglobosin A exhibited a significantly different level of decrease after treatment with acid or alkali solutions in comparison with the control groups. Chaetoglobosin A was more stable in the alkaline environment ($13 \geq pH \geq 9$), but showed significant degradation when the alkalinity was too strong (pH = 14). Under the condition of no direct contact, chaetoglobosin A could tolerate a weak acid environment ($pH \geq 2$), but decreased sharply when the pH value was reduced to 1. These results also suggested that the organic acids in the medium may destroy chaetoglobosin A when using alcohols as the solvent.

Moreover, deacidification experiments revealed that proper alkali solutions were beneficial to reducing the impurities. The use of a 0.1 M sodium hydroxide solution (pH = 13) showed a positive effect on purification. Comparison of the amounts of chaetoglobosin A between the control and experimental groups indicated that deacidification treatment could increase the content of chaetoglobosin A from 4.82% to 6.15%.

The stability of chaetoglobosin A had not been tested completely,[10] and this was the first report on the pH stability of chaetoglobosin A. The results showed that acids with a low pH value would sharply destroy chaetoglobosin A, suggesting that the organic acids in the crude extract should be wiped off as much as possible before further purification steps, and that suitable concentrations of sodium hydroxide solution could effectively serve as a protection reagent in combination with ethyl acetate. Owing to its weak stability in acid and lye, the typical process of β-lactam antibiotics in adjusting pH and reverse extraction does not appear to be suitable for the purification of chaetoglobosin A.[26]

Purification of the crude extract

A low content has been one of the main restrictions for the application of crude chaetoglobosin A extracts. Therefore, certain reagents, including n-hexane (used for degreasing), sodium hydroxide solution (used for deacidification), and methanol-water (used for degreasing), were tested for their ability to effectively remove these impurities. Fig. 4 shows the results of these tests.

n-Hexane was confirmed to be incapable of extracting chaetoglobosin A in the present study, confirming the results of a previous study.[24] Hence, samples extracted from the fermentation residue of 1 g of cornstalk medium were dissolved in methanol, and the extraction was carried out in both n-hexane and methanol with the same volume used for degreasing. However, the result was not desirable. In particular, n-hexane failed to increase the content of the product, although it did not reduce the amount of chaetoglobosin A.

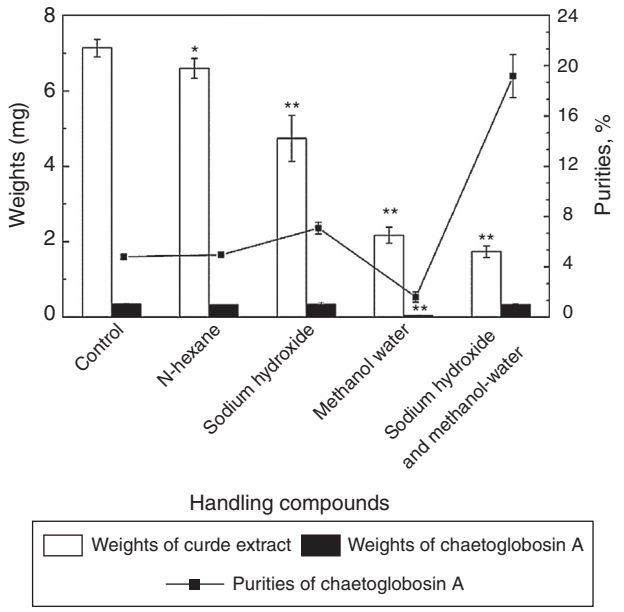

Fig. 4 – Effects of different compounds on the purification of chaetoglobosin A. The initial amount of chaetoglobosin A in every sample was 0.5 mg. Asterisks indicate a significant difference between the results of the treatment groups and control ($^{*}p < 0.05$; $^{**}p < 0.01$).

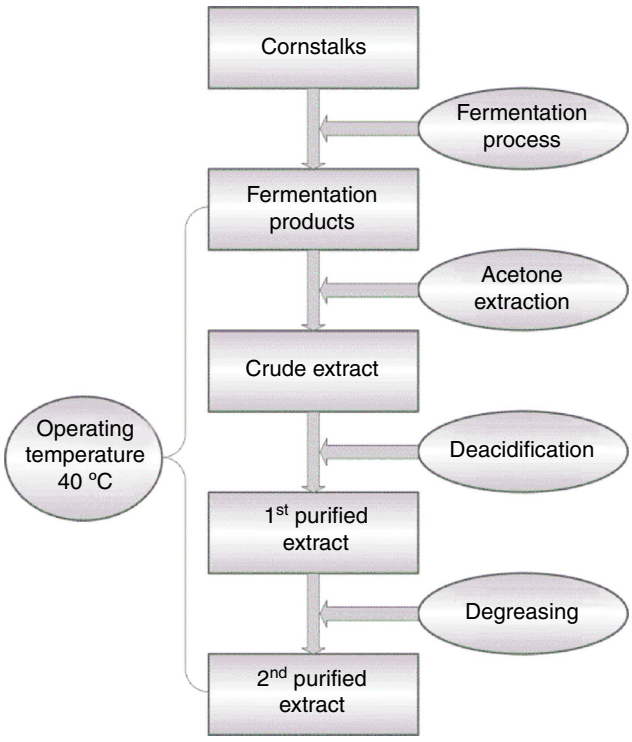

Fig. 5 – Production process of chaetoglobosin A products. Rectangles represent compounds, and ovals represent processes.

In the previous study, sodium hydroxide solutions were confirmed to be effective for purification with little decrease of chaetoglobosin A,[24] and this result was confirmed in the present study. However, there were still large amounts of impurities remaining in the crude extracts. By contrast, although methanol-water removed most of the impurities, the amount of chaetoglobosin A also decreased significantly, which was due to the organic acid in the samples. Collectively, these results showed that the combination of alkali liquors and methanol-water had the best effect on purification, and the content reached 19.17 ± 1.72% without a decrease in the amount of chaetoglobosin A. Therefore, the combination of alkali liquors and methanol–water was chosen as the optimal purification procedure of the crude extract.

Establishment of the complete production process

Based on the above results, an optimal production process for chaetoglobosin A was established. A schematic of the whole process is shown in Fig. 5. After a series of purification steps under the optimal extraction conditions, the contents of chaetoglobosin A in the cornstalk-fermentation products almost quadrupled compared to the original value (19.17% vs. 4.80%). This content was sufficient for the development of agricultural antibiotic products.

In previous studies, the crops were the main substrates used for the microbial synthesis of chaetoglobosin A, but only a few of reports have provided data related to the yields and contents in the crude extract (Table 1). As shown in Table 1, potato was a frequently used substrate with high outputs of chaetoglobosin A. Ethyl acetate and chloroform were the most widely used but benzyl-ethanol solution displayed the best extraction effect. In this study, by comparing the effects of different solvents, we found that acetone was the optimal extractant for the cornstalk fermentation residues. This finding was applied for potato fermentation, and the data showed that the contents of chaetoglobosin A in the crude extract also increased.

C. globosum was cultured on PDA and subjected to the newly established production procedure to verify the method. After applying the production process outlined in Fig. 5, the amount of chaetoglobosin A in the fermentation residue of 200 g potatoes (1 L PDA medium) was 59.88 ± 4.22 mg and the content increased from 11.15 ± 0.37% (original value of the crude extract) to 20.35 ± 0.55%. There was no significant ($p = 0.32$) difference in the quality of the products fermented from potatoes and cornstalks (content 19.17 ± 1.72%). Together, these results showed that cornstalks could be used to replace potatoes in the production of chaetoglobosin A using our proposed production process. Moreover, the significant increase of the initial content of chaetoglobosin A may also contribute to reducing the operating steps and costs of chromatographic purification. In a word, the new production process in Fig. 5 could be applied as a preliminary sample preparation step.

Biological activities of products against R. solani

The ultimate objective of preparing products with abundant chaetoglobosin A was to search for a new potential agricultural fungicide. Toward this end, the biological activities of the samples were evaluated against a phytopathogen, and the fungus *R. solani* was chosen for this evaluation in this

Table 1 – Chaetoglobosin A production by different strains of *Chaetomium*.

Substrate	Strain	Extraction solvent	Yield[a]	Content[b]	Study
Potato-glucose	*C. globosum* NK102	$CHCl_3$	50.5 mg/L	No data	[4]
Potato-glucose	*C. globosum* 68-SA-2	Benzene-ethanol	No data	11.6%	[7]
Rice		$CHCl_3$	No data	9.0%	
Oat-maltose	*C. globosum* No.05	Ethyl acetate	26.44 mg/L	4.81%	[8]
Oat-maltose	*C. globosum* No.04	Ethyl acetate	5.24 mg/L	0.73%	[9]
Potato-glucose	*C. globosum* NK102	$CHCl_3$	53.71 mg/L	No data	[19]
Corn-sucrose	*C. globosum* DAOM240349	Ethyl acetate	33.13 mg/L	No data	[22]
Potato-glucose	*C. globosum* W7	Acetone	59.88 mg/L	11.15%	This study
Cornstalk	*C. globosum* W7	Acetone	0.34 mg/g substrate	4.80%	This study

[a] Yield refers to the yield of chaetoglobosin A in the culture system.
[b] Content refers to the content of chaetoglobosin A in the crude extract.

study. Owing to the weak water solubility of chaetoglobosin A, ethanol was chosen as the solvent given its beneficial characteristics of low cost and low toxicity. Colony area was used as the evaluation criterion for an inhibition effect, because the control of fungal diseases was mainly targeted at preventing the spread of the infection area.[8] Although chaetoglobosin A was previously shown to be the main antifungal component in the crude extract,[8] in some cases, the use of different experimental materials may lead to different results. Hence, we designed a comparative experiment to evaluate the difference in the antifungal effect between the crude extract and pure chaetoglobosin A to verify the opinion put forward in the previous study. The inhibitory efficiency of the crude extract against *R. solani* is shown in Fig. 6.

In this test, water was set as the blank control, and ethanol was used as the solvent of chaetoglobosin A to reach different final concentrations. As shown in Fig. 6A, there was a statistically significant difference ($p < 0.05$) in the average colony area of *R. solani* between the water control and the ethanol group, which contained no chaetoglobosin A. As ethanol presented significant inhibition, the ethanol group was set as the solvent control in the calculation of the mycelial inhibition rate. The inhibition ratios of colony areas were calculated according to the following equation: mycelial inhibition (%) = $[(dm - dt)/(dm - di)] \times 100\%$, where dm is the mean colony area of the ethanol control set, dt is the mean colony area of the treatment set, and di is the initial colony area of the fungal mycelium inoculated. With increasing concentration of chaetoglobosin A from 0.5 μg/mL to 5.0 μg/mL, the average areas of *R. solani* mycelia decreased significantly ($p < 0.05$), and the percentage of the inhibition of *R. solani* growth also increased significantly simultaneously. In the treatments with higher concentrations, the inhibition effect occurred earlier. In the first 72 h, mycelia showed non-significant growth as the concentration of chaetoglobosin A increased from 25 μg/mL to 50 μg/mL, but in the subsequent 48 h, disinhibition was observed and the mycelia began to grow quickly. The IC_{50} value for chaetoglobosin A on *R. solani* mycelial growth increased from 1.15 μg/mL to 4.39 μg/mL, which was calculated based on the regression curve $Y = a \times \log_{10}{}^{(X)} + b$, determined by probit analysis,[27] where Y is the colony area, X is the concentration of chaetoglobosin A, and a and b are the constants obtained at different time points (Table 2). At the 48th hour, the IC_{50} value tended to be stable and the mean value was about 3.88 μg/mL.

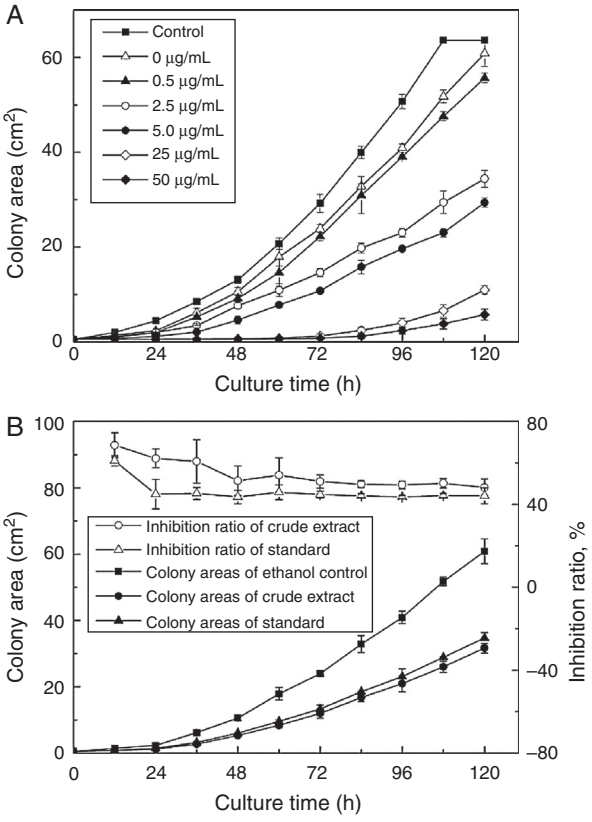

Fig. 6 – Inhibitory efficiency of the crude extract (20% content) against *Rhizoctonia solani*. (A) Inhibitory efficiency of chaetoglobosin A at different final concentrations under different culture times. (B) Verification of the predicted IC_{50} value of chaetoglobosin A using ethanol as the solvent, and comparison of the effect of the ethanol standard under the same concentration (3.88 μg/mL).

The mean IC_{50} value was 3.88 μg/mL, and this concentration was used in the subsequent verification test. In comparison with the control groups, the colony areas of *R. solani* decreased significantly, and the inhibition ratio increased from 48.22% to 54.15% after 36 h (Fig. 6B). The standard had a slightly lower effect with the same amount of chaetoglobosin A compared to the crude extract, with an inhibition ratio of approximately 44%. These results showed

Table 2 – Inhibitory regression curves and predicted IC_{50} values of chaetoglobosin A against *R. solani* at different culture times.

Culture time (h)	Regression curve[a]	R^2	IC_{50} (μg/mL)
12	$Y = 34.975 \times \log_{10}(X) + 47.876$	0.9417	1.15
24	$Y = 47.939 \times \log_{10}(X) + 23.234$	0.8761	3.62
36	$Y = 43.620 \times \log_{10}(X) + 33.399$	0.9656	2.40
48	$Y = 48.260 \times \log_{10}(X) + 22.725$	0.9496	3.67
60	$Y = 44.902 \times \log_{10}(X) + 27.640$	0.9760	3.15
72	$Y = 48.658 \times \log_{10}(X) + 21.829$	0.9863	3.79
84	$Y = 48.026 \times \log_{10}(X) + 20.852$	0.9896	4.05
96	$Y = 46.474 \times \log_{10}(X) + 21.437$	0.9863	4.12
108	$Y = 43.525 \times \log_{10}(X) + 23.974$	0.9906	3.96
120	$Y = 41.306 \times \log_{10}(X) + 23.462$	0.9936	4.39

[a] X represents the concentration of chaetoglobosin A, and Y indicates the inhibition ratio against *R. solani*.

that the difference observed between the two kinds of samples was not statistically significant ($p > 0.05$), which indicated that chaetoglobosin A was mainly responsible for the inhibitory action. The IC_{50} value of chaetoglobosin A against *R. solani* suggested that this compound shows good potential as a natural protectant with long-term and stable efficacy (at least 5 days).

Conclusion

This study is the first to report the application of cellulose substrates in the production of chaetoglobosin A. Our results demonstrated that cornstalks could be effectively used to produce chaetoglobosin A, providing distinct advantages of a low substrate cost and good availability, and the quality of products was greatly increased by using the optimized production process developed in this study. The product retained biological activity and the content reached up to about 20%, which is sufficient for the development of a biopesticide. Furthermore, the percentage of crude protein in the fermentation residue was increased by 37.92% in comparison with the original cornstalks, and the extraction residue can be used as animal feed, making the production process even more economical. The possibility of developing a cornstalk biotransformation process for the large-scale production of chaetoglobosin A would result in substantial cost reduction by using already available agricultural wastes.

Conflicts of interest

The authors declare no conflicts of interest.

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