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Purification and quantification of destruxins A and B from *Metarhizium anisopliae*

Jech-Wei Chen, Bing-Lan Liu, Yew-Min Tzeng*

Institute of Biotechnology, National Dong Hwa University, 1 Sec. 2, Da Hsueh Road, Shoufeng, Hualien, Taiwan

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Abstract

Purification of insecticidal cyclodepsipeptides, destruxins A (DA) and B (DB), from *Metarhizium anisopliae* fermentation broth was performed. The isolation scheme of these destruxins using ion-exchange chromatography, silica gel chromatography, and semi-preparative HPLC chromatography is presented. The quality of the semi-preparative products was unique. Over 90% purity (based on HPLC chromatograms) was achieved for both DA and DB. Purified destruxins were further identified employing the ^{1}H NMR and fast atom bombardment MS methodology. The HPLC quantification methods for DA and DB in the fermentation broth have been established. The use of the purified destruxins as analytical standards demonstrated good correlation.

Keywords: *Metarhizium anisopliae*; Destruxins; Mycotoxins; Toxins; Peptides

1. Introduction

Pathogenic fungi use different strategies to penetrate the outer surface of their targets and kill them. One important aspect of the mode of action of these pathogens is the production and release of mycotoxins from these pathogens [1]. Among the metabolites produced by entomogeneous fungi, the destruxins (Dtxs) are of particular interest because they are the only mycotoxins detected in the insect body at advanced stages of infection to cause death [2]. Over 20 different structurally related destruxins have been isolated, and 15 of these were originally isolated from *Metarhizium anisopliae* [3]. A typical structure of destruxins is depicted in Fig. 1. Accordingly, some destruxins have been shown to posses an immunodepressant activity in insect model systems, and cytotoxic and cytostatic effects on mouse leukemia cells [4]. Furthermore, several destruxins also showed a strong suppressive effect on the production of the hepatitis B surface antigen (HBsAg) in human hepatoma cells [5]. To date, over 25 destruxins have been isolated and identified [6–10]. Among them, destruxins A, B, and E are the most abounding components.

For the mass production of these useful components, the submerged fermentation process is the best choice [11]. An accurate quantitative methodology is pivotal in many aspects of destruxin studies. Thus, the production of a pure analytical standard becomes the first challenge prior to any cultivation studies. Although several research groups have employed different methods for the isolation of destruxins from culture broth extracts [8,12,13], a

*Corresponding author. Tel.: +886-3-866-2500 ext. 1874; fax: +886-3-866-2051, e-mail: ymtzeng@cc.ndhu.edu.tw

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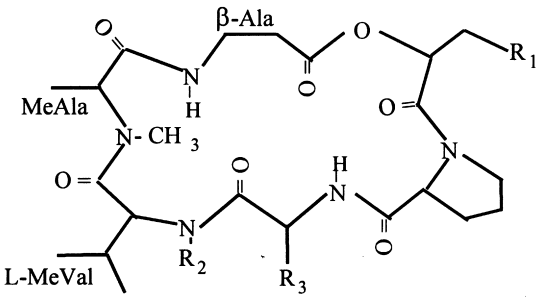

R_1	R_2	R_3	Destruxin
-CH=CH_2	-CH_3	-$CH(CH_3)CH_2CH_3$	A
-CH(CH_3)(CH_3)	-CH_3	-$CH(CH_3)CH_2CH_3$	B
-CH(CH_2OH)(CH_3)	-CH_3	-$CH(CH_3)CH_2CH_3$	C
-CH(CHOOH)(CH_3)	-CH_3	-$CH(CH_3)CH_2CH_3$	D
-CH-CH_2 (O)	-CH_3	-$CH(CH_3)CH_2CH_3$	E
-CH(CH_3)(CH_3)	-H	-$CH(CH_3)CH_2CH_3$	DMDB
-CH=CH_2	-CH_3	-$CH(CH_3)_2$	A_2
-CH-CH_2 (O)	-CH_3	-$CH(CH_3)_2$	E_2

Fig. 1. Representative structure of destruxins.

concrete analytical protocol is still unavailable. In this work, the isolation and characterization of destruxins A (DA) and B (DB) from *M. anisopliae* by means of semi-preparative high-performance liquid chromatography (HPLC) quantification, and of nuclear magnetic resonance (NMR) and fast atom bombardment mass spectrometry (FAB-MS) qualitative identification is described.

2. Experimental

2.1. Microorganism and cultivation

Metarhizium anisopliae var. *anisopliae* (Metschnikoff) was kindly supplied by Dr. S.S. Kao, Taiwan Agricultural Chemicals and Toxic Substances Research Institute (Wufeng, Taiwan). The spore suspension used as inocula was obtained from five-day-old submerged cultures grown on 3.5% Czapek-Dox (CD) broth (Difco, Detroit, MI, USA) supplemented with 0.5% bactopeptone (Difco), at 200 rpm and 28°C. For submerged fermentation, 5% (v/v) inocula level was used. The cultivation was performed with a 500-ml Erlenmeyer flask containing 100 ml of 3% maltose (Nihon Shiyaku, Osaka, Japan) and 0.5% bactopeptone liquid medium and the culture was allowed to grow at ambient temperature (28°C) for 14 days on a rotary shaker (150 rpm). Samples were filtered through a 0.22-μm membrane disc before HPLC analysis.

2.2. Isolation and purification of destruxins

The destruxins A and B used in this study were isolated and purified from submerged culture in 3% maltose containing 0.5% bactopeptone. After 14 days on a gyro-rotary incubator at 150 rpm and 28°C, the liquid culture was blended for 30 s. The mycelium and impurities were removed from fermentation broth by centrifugation (8000 *g*, 30 min). The supernatant was then extracted three times with equal volume of methylene dichloride. The organic layer was collected, and concentrated with a vacuum rotary evaporator (Model N-1NW, Eyela, Tokyo, Japan). This crude extract was then passed through an Amberlite (Rohm & Haas, Philadelphia, PA, USA) TRA-400 CP cation-exchange chromatographic column (300×15 mm) and eluted with distilled water under gravity mode. The elute was directly loaded into another Amberlite IR 120 Plus anion-exchange column (300×15 mm) without further treatment. This column was also washed with distilled water under gravity mode. Pooled eluted sample was redissolved in an equal volume of acetonitrile, and concentrated using a vacuum rotary evaporator.

Acetonitrile-soluble residue was flash chromatographed on a Merck (Darmstadt, Germany) K-60 silica gel column (230–400 mesh, 240×25 mm) employing a 50-ml step-wise methylene dichloride–methanol (95:5, at the beginning) solvent gradient to give desired polarities. Further purification was performed on a semi-preparative RP-C_{18} HPLC column (Merck LiChrosorb, 7 μm, 250×10 mm). A typical running gradient was as following: 0 min (0% acetonitrile)→30 min (40% acetonitrile)→40 min (50% acetonitrile)→60 min (50% acetonitrile). The eluting solvent with a flow-rate of 3 ml/min was employed. HPLC-grade acetonitrile was purchased from Mallinckrodt Baker (Paris, KY, USA) and the water used for HPLC was obtained from a Millipore (Bedford, MA, USA) Milli-Q water system. All solvents used were degassed and filtered through a 0.22-μm filter before use. Fractionated samples were characterized by means of FAB-MS and ^{1}H NMR

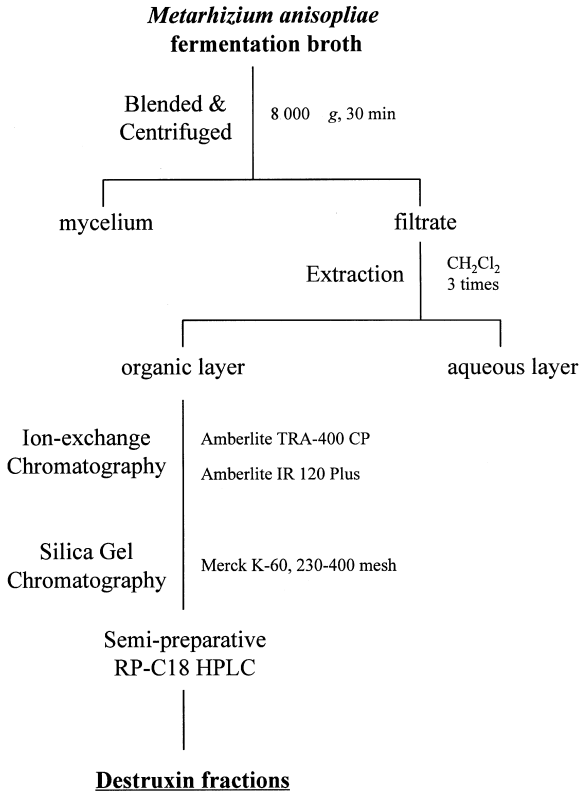

Fig. 2. The purification scheme of *M. anisopliae* fermentation broth.

spectrometry. The scheme for isolation and purification of destruxins is shown in Fig. 2.

2.3. MS and NMR spectrometry

All MS analyses were performed on a JMS-HX 110 (Jeol, Tokyo, Japan) double-focusing magnetic sector mass spectrometer having modified Nier–Johnson geometry. The instrument was operating at 10 kV accelerating voltage and a nominal resolution setting of 3000. Glycerol or thioglycerol (TG) was used as a matrix. Spectra were recorded using the Joel DA5000 data system. Destruxin samples for NMR studies were unbuffered by vacuum-drying and reconstituted in the C^2HCl_3. ^1H NMR spectra were recorded at 300 MHz on Bruker (Billerica, MA, USA) DPX 300 spectrometer. Data were processed by UXNMR software on a Silicon Graphics Indy workstation.

2.4. HPLC analysis

The analytical HPLC system consisted of a Hitachi (Tokyo, Japan) L-7100 pump; a 20-μl fixed loop, and a Model L-7400 variable-wavelength UV–Vis detector. For destruxin analysis, a Micra (Northbrook, IL, USA) NPS RP-C_{18} analytical column (33×4.6 mm) was employed. A gradient combination of acetonitrile–water was employed as follows: 0 min (0% acetonitrile)→20 min (27% acetonitrile)→25 min (90% acetonitrile)→30 min (90% acetonitrile). The eluting solvent with flow-rate of 1 ml/min was fixed throughout the studies. Injection volume was 5 μl, and the detector monitored absorption at 215 nm for a period of 30 min.

3. Results and discussion

The medium used in the submerged culture studies

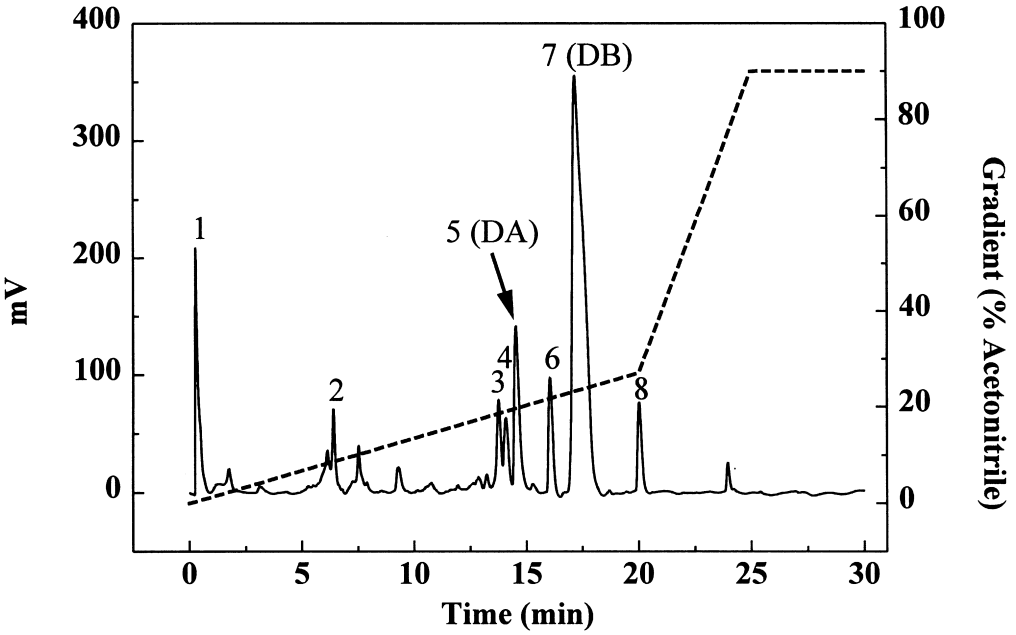

Fig. 3. HPLC chromatographic plot of *M. anisopliae* cultivation broth on an NPS RP-C_{18} column. A non-porous silica analytical column (33×4.6 mm) was used for quantification analysis. The sample was eluted with a linear gradient of 0 min (0% acetonitrile)→20 min (27% acetonitrile)→25 min (90% acetonitrile)→30 min (90% acetonitrile) at 1 ml/min flow-rate. The dashed line shows the gradient profile. The absorbance was detected at 215 nm. Significant absorption peaks were numbered. Among them, peak 1 was attributed to the solvent peak, whereas peak 2 is more likely to be the DE. Peaks 3, 4, 6 and 8 were belived to be the other destruxin derivatives. All these unspecified dextruxins are still under investigation.

of *Metarhizium anisopliae* has been focused on the combination of CD broth and peptone with no exception in the literature. In this study, a modified medium was used to grow this fungus. *M. anisopliae* was cultured on a maltose medium containing 0.5% peptone in a shaking flask. Using the maltose as a main carbon source gave a higher destruxin yield than that of the conventional CD broth medium. Mycelium from the 14-day-old culture of *M. anisopliae* was blended, and separated by centrifugation. The supernatant was extracted three times by equal volume of methylene dichloride. A method for the separation and purification of destruxins A and B were followed, as shown in Fig. 2. The sample was analyzed by an analytical RP-C_{18} HPLC column as described in Section 2.4. Fig. 3 shows the HPLC chromatogram of the crude extract. Separation of the mixture of various destruxins in the culture medium has been achieved with a water–acetonitrile gradient over a period of 30 min. Extracts of fermentation

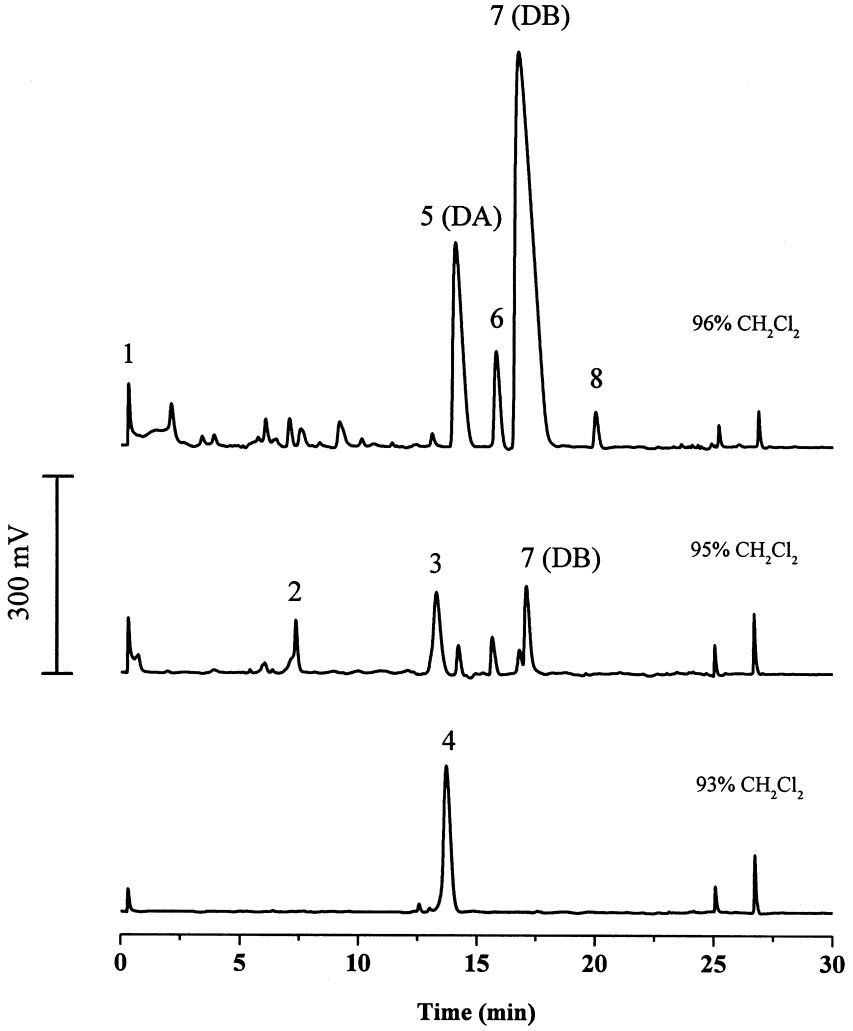

Fig. 4. HPLC chromatograms of silica gel fractions at 93, 95 and 96% (v/v) methylene dichloride. The samples were taken from different eluting fractions (see Section 2.2 for details). Analysis was performed on an analytical HPLC column, same as in Fig. 3. Peak numbering as in Fig. 3.

broth could contain many destruxins and metabolites [10,14]. As shown in Fig. 3, destruxin peak 7 (~17 min) has highest intensity and peak area. Three close peaks (peaks 3–5) were centered around 13 to 15 min. With the elution gradient employed in this study, most of the peaks appeared within 20 min after injection. The other destruxins did not show a significant intensity due to a relatively low concentration in the crude extract.

The sample was further separated by two-step ion-exchange chromatography. Combination of anionic and cationic exchange provided an another step to removing charged impurities existed in the mixture. The crude extract collected from ion-exchange column was subjected to flash silica chromatography as a prepurification step. The column was eluted with a mixture of methylene dichloride–methanol (98:2), and then with increasing proportion of methanol in the mixture until 7%. Fig. 4 shows fractionated HPLC chromatographic plots for the three concentrations of methylene dichloride. Most of peaks were washed out at the methylene dichloride level of 96%. Peak 1 (~1 min, cf. Fig. 3) is decreased dramatically after ion-exchange chromatography and thereafter. In practice, two major destruxins (DA and DB) were isolated from the first silica gel fraction. As evident from the chromatogram these destruxins showed excellent resolution on RP-C_{18} HPLC under the experimental conditions. In addition, peaks 3–5 (Fig. 3) were separated by different polarity solvent gradient as determined from Fig. 4. The results suggested that the conventional one-step or one-polarity elution is not suitable for the separation of those destruxins by the silica gel column. The similar structure molecules, therefore, have the better resolution as resulted from the proper polarity employed at this step.

Further purification was done by loading the sample from silica gel chromatography into semi-preparative HPLC column. The elution delivered from a single peak were collected and concentrated. Fig. 5 shows the chromatogram plot of a semi-preparative HPLC fraction. The retention profile of the destruxins is different from that of the analytical HPLC column. This is due to the higher sample concentration (also high injection volume) which caused overlapping. The highest peak appeared at the 40 min after injection. This peak represented DB

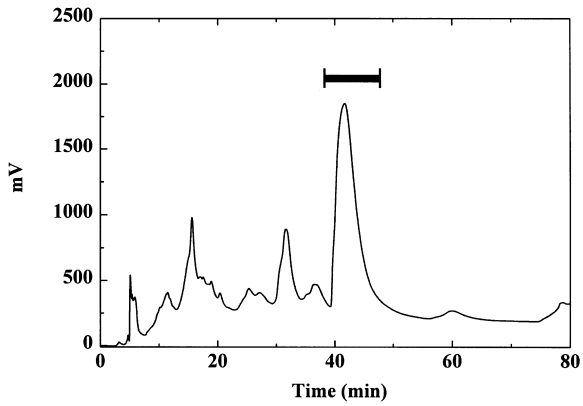

Fig. 5. Semi-preparative reversed-phase HPLC chromatographic plot of the destruxin sample. Partial purified sample was injected into a semi-preparative reversed-phase C_{18} HPLC column. Chromatographic gradient was: 0 min (0% acetonitrile)→30 min (40% acetonitrile)→40 min (50% acetonitrile)→60 min (50% acetonitrile). The eluting solvent with a flow-rate of 3 ml/min was employed, and the absorbance was recorded at 215 nm. The bar indicates where a single destruxin was collected from eluted fractions.

(t_R=~17 min) as confirmed by physicochemical and spectral data (see below). Similarly, DA could be purified in the same manner according to the procedure described above. Chromatographic plots of purified DA and DB are given in Fig. 6. The retention times for DA and DB were 14.86 and 17.6 min, respectively, corresponding to peaks 5 and 7 found in the Fig. 3. Only some tiny impurity peaks were observed in both cases, implying that the impurities scarcely existed. Over 90% purity was obtained for both DA and DB. This was calculated based on the total HPLC chromatogram peak area determined at 215 nm where the response coefficient for all presented components was assumed to be equal.

The identities of these purified destruxins were established on the basis of FAB-MS and ^{1}H NMR spectrometry. Some destruxins have been investigated by NMR spectrometry in a solvent system similar to that used in this study. Fig. 7 represents the ^{1}H NMR spectrum for purified destruxins A and B. The chemical shift appearing at 7.2 ppm both in Fig. 7A Fig. 7B was ascribed to the C^2HCl_3. Carefully comparing those two spectra in Fig. 7, the significant differences were found at 5.35 ppm and in the region 0.7–1.4 ppm, respectively. DA exhibited a

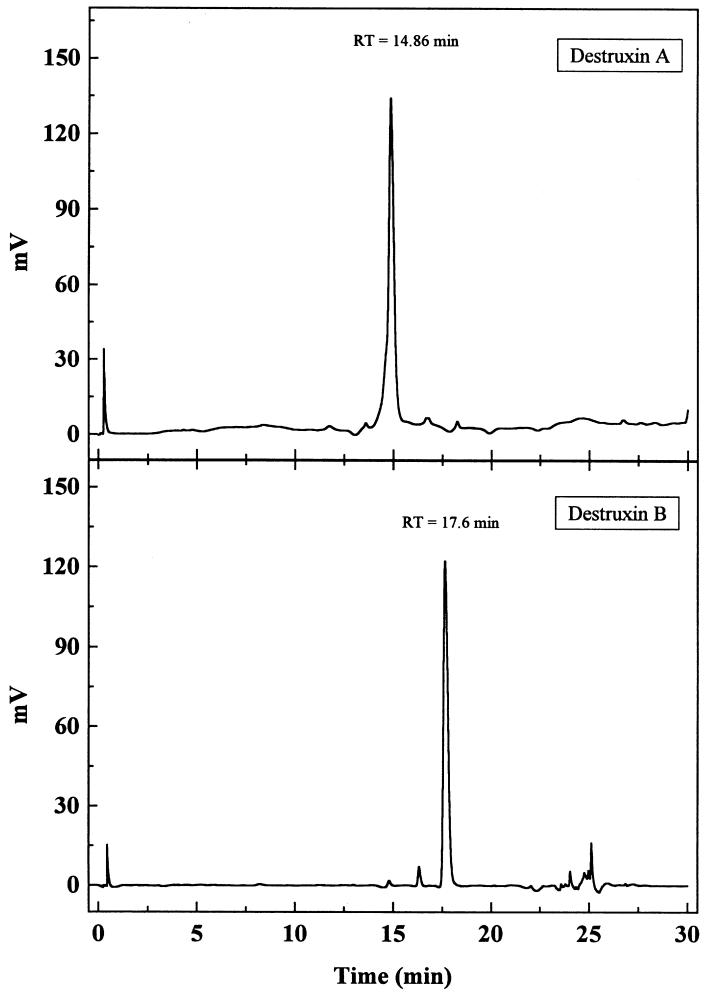

Fig. 6. HPLC analysis of purified DA (top) and DB (bottom) from semi-preparative HPLC. Chromatographic conditions as in Fig. 3. RT=Retention time.

strong peak between the olefinic protons of the pentenoic acid moiety and appeared in the region 5.1–5.5 ppm as seen in Fig. 7A. In addition, the methyl triplet (isoleucine $-CH_2CH_3$) resonates at 0.83 ppm and other peaks around this region, found in Fig. 7B, suggest that this compound is DB as described by Gupta et al. [14]. The profiles of the present spectra for DA and DB are essentially identical to those reported in the literature [14,15]. However, there is an artifact at about δ 2.5 in Fig. 7A or δ 6.1 in Fig. 7B, which may be the result of a number of factors such as field inhomogeniety, or impurities. Fig. 8 shows the normal positive FAB-MS spectrum of semi-preparative HPLC fractions of the soluble destruxins A (molecular mass~577) and B (molecular mass~593.7) produced from cultivation of *M. anisopliae*. Purified fraction from the culture gave a peak of m/z 578 (M+1) corresponding to DA (Fig. 8A). Moreover, a very strong peak appearing in the position of m/z 594 corresponds to DB (Fig. 8B). Although DE has a similar molecular mass, its structure is quite different in the substitution group [8,13]. Comparison of the retention time on HPLC also suggested that no DE peak was obtained. Similarly, DA shows protonated molecules at m/z 577, which is similar to the data reported elsewhere

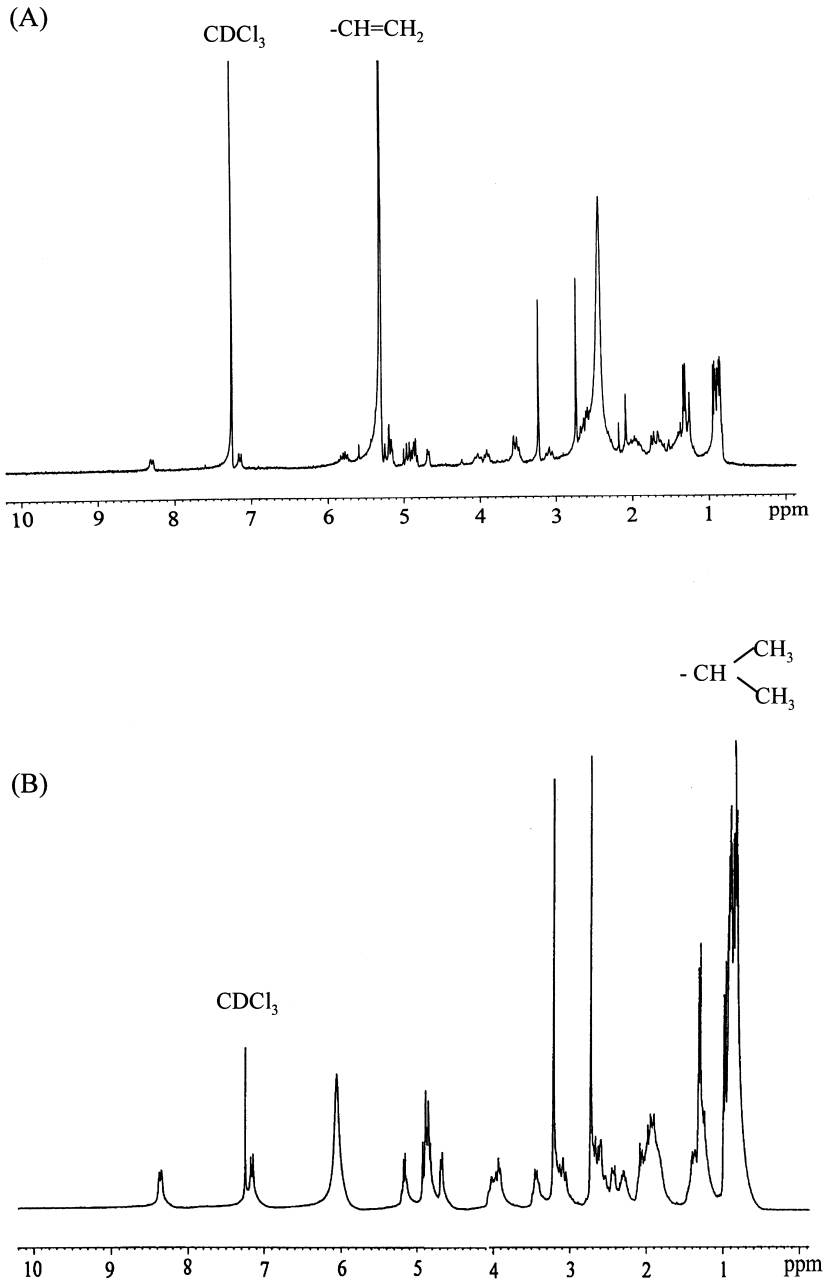

Fig. 7. ^{1}H NMR spectra of the purified DA (A) and DB (B).

[16]. Thus, the results of the mass spectral analysis, coupled with the ^{1}H NMR data confirmed peak 5 in Fig. 2 as DA and suggested peak 7 as DB. Additionally, combination of HPLC, FAB-MS and ^{1}H NMR analysis showed that the isolated destruxins were quite pure. The purities of both DA and DB were over 90% (see above).

Good correlation on the concentration dependence experiments was also demonstrated for these purified destruxins (Fig. 9). The concentration of destruxin

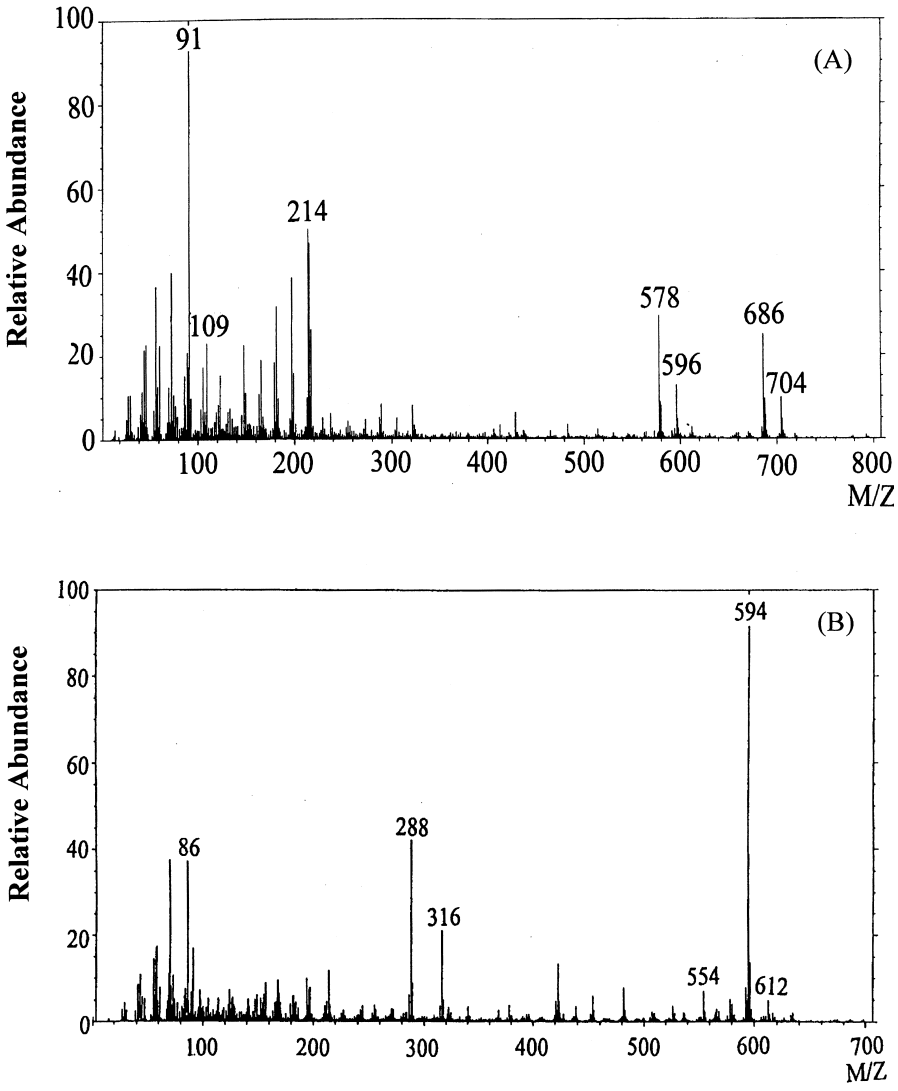

Fig. 8. The normal FAB-MS spectrum of purified DA (A, molecular mass~577) and DB (B, molecular mass~593) produced by *M. anisopliae*.

(in mg/ml), therefore, can be calculated from the equation derived from the least-square fitting of the standard curve as follows: [Destruxin A]=$1.845\times 10^{-7}\times$peak area$-0.027$. Similarly, [Destruxin B]=$2.294\times 10^{-7}\times$peak area$-0.014$.

The concentration ranges which could be adapted to the above equations were 0–0.2 and 0–2 mg/ml for DA and DB, respectively.

Although the HPLC methodology is widely utilized in many reports, only a few workers focused on the quantification of destruxins [13]. The concentration of destruxins A and B in the culture broth (cf. Fig. 3) were analyzed by HPLC, using a non-porous silica packing without pretreatment or concentration. Accordingly, the concentrations of DA and DB in a batch culture were 5.18 and 33.67 mg/l, respectively. These analytical results demonstrated that the purified destruxins could be used to construct a HPLC calibration curve for the determination of destruxins in a cultivation mixture. Furthermore, DB

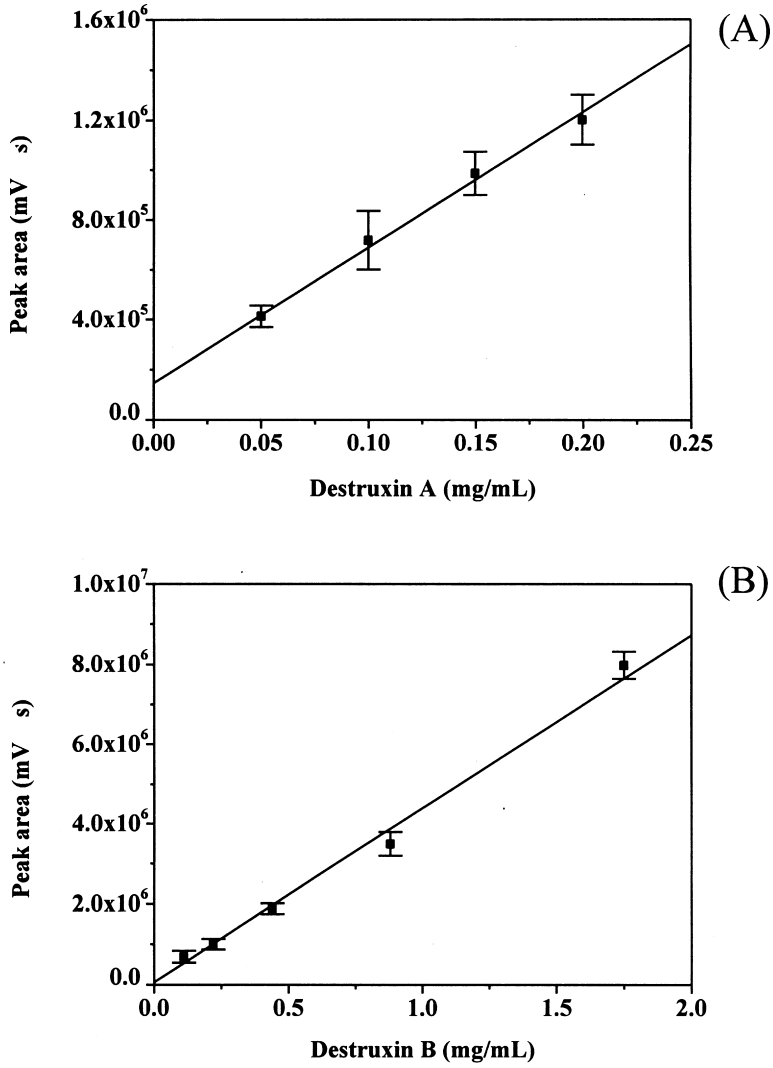

Fig. 9. The standard curves of the purified DA (A) and DB (B). Each point represents the mean value from four different determinations.

was found to be the most abundant component in the strain used in this study. Abundant species of the destruxins is known to vary from case to case, depends on the strains used and cultivation conditions.

4. Conclusions

The purification technique described in this report is very straightforward and is suitable for laboratory-scale operation. With the processes described herein it can produce enough pure destruxins for quantification purposes, and for further detailed studies of these destruxins. Rather pure destruxins (DA and DB) could be obtained and for the components of interest it is preferred over quantification by means of HPLC methodology. Consequently, analysis of destruxins from the culture broth could be accomplished by injection of the filtered sample into the HPLC system directly without other pretreatment or concentration. The purified DA and DB were also confirmed by FAB-MS and ^{1}H NMR spectrometry. Although the other destruxins (especially DE) were

not present in sufficient amounts for isolation in this study, the isolation scheme still provided a useful and straightforward methodology on purification of cyclodepesipeptide toxins.

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