Research Article
Application of TLC in the Screening of Acarbose-producing Actinomycetes

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Abstract: Acarbose is widely used in medicine, such as the treatment of diabetes and obesity. A simple Thin Layer Chromatography (TLC)-scanning technique was developed for the rapid and accurate analysis of acarbose in a large number of fermentation broths of actinomycetes to screen for acarbose producer. The linearity of the acarbose in this way was good within the range from 2 to 10 μg (r² = 0.9997). This technique didn’t need expensive instrument and complex procedure for the detection of acarbose in the fermentation broths. An actinomycete with acarbose yield of 1.83 mg/mL was obtained. The results demonstrated that TLC-scanning was a cheap and simple technique for the accurate screening of acarbose-producing actinomycetes.

Keywords: Acarbose, actinomycetes, diabetes, mellitus, screening, TLC

INTRODUCTION
Diabetes mellitus is a worldwide chronic disease that is caused by an imbalance of glucose homeostasis. If it is not controlled, diabetes can lead to serious chronic complications in the eyes, kidneys, peripheral nerve system and arteries and result in impaired quality of life, disability and mortality (Yang et al., 2010). Acarbose is a pseudo-oligosaccharide, which acts as a competitive α-glucosidase inhibitor. The mechanism of inhibition for these enzymes can be due to the cyclohexene ring and the nitrogen linkage that mimics the transition state for the enzymatic cleavage of glycosidic linkages (Yoon and Robyt, 2002). Acarbose, as an oral drug used in the therapy of type II diabetes owing to its indigestibility and nearly undetectable toxicity, was first launched in Germany in 1990 and had been successfully marketed worldwide (Li et al., 2012). Treatment with acarbose has been shown to prevent or delay the onset of type II diabetes, high blood pressure and cardiovascular complications among individuals with impaired glucose tolerance (Chiasson et al., 2002, 2003).

Comparatively, synthesis by microorganisms is an effective strategy to produce cost-effective α-glucosidase inhibitor. Actinomycetes have received much attention for their capacity to produce clinically important antibiotics and other biologically active secondary metabolites (Choi et al., 2005). It has been reported that some actinomycetes, including species of Streptomyces (Iwasa et al., 1970), Actinoplanes strains SE 50 (Lee and Egelkrout, 1998; Schmidt et al., 1977) and Actinoplanes utahensis ZJB-08196 were able to synthesize acarbose (Wang et al., 2012).

In previous reports, potential acarbose producers were screened using the iodine-starch colorimetry method or 3, 5-dinitrosalicylic acid (DNS, Bernfeld method) (Miller, 1959). The iodine-starch colorimetric method was time-consuming, semi-quantitative and the broad inflection point always led to inaccuracies. The Bernfeld method needed a boiling water bath to maintain the reaction between DNS and reducing sugar, which prevented it from being conducted in a 96-well microtiter plate format. Feng et al. (2011) developed a colorimetric method to screen for α-amylase inhibitor producing strains, which based on enzymic catalytic reaction. In the above methods, however, the enzymes in the fermentation broths, such as α-amylase and α-glucosidases, might involve in catalyzed reactions and cause inaccuracies. Furthermore, the metal ions in the fermentation broth would accelerate or inhibit the enzymatic reactions and affect the accuracy of acarbose detecting. In addition, these techniques usually required either complicated procedures or sophisticated equipment for the analysis of acarbose.

The TLC method had several advantages including the lower cost, less rigorous sample preparation, the ability to analyze multiple samples simultaneously and the ease of visualization. So it was widely used to separate and quantify for many substances (Kongkiatpaiboon et al., 2013; Abdelaleem and Abdelwahab, 2013; Sahana et al., 2011). In the present
study, we developed a simple, non-expensive and fast TLC technique that was sensitive and accurate. The new method had enabled us to screen for high-acarbose-producing actinomycetes from the massive samples in a short time.

**MATERIALS AND METHODS**

**Materials:** Acarbose standard was purchased from Sigma. Silica TLC plate (TLC Silica gel 60 F254, 0.20 mm thickness) was obtained from Merck KGaA, Germany. Glucose, sucrose, maltose, glycerol, monosodium glutamate, K₂HPO₄·3H₂O, FeSO₄·7H₂O, MgSO₄·7H₂O, NaOH, K₂CrO₇, CaCO₃, acetone, n-propanol, diphenylamine, aniline and phosphoric acid were analytical reagent. Peptone, agar, soybean meal and yeast extract were obtained from commercial sources.

**Media:** The agar medium used for isolating actinomycetes was consisted of (g/L): starch, 15.0; glucose, 10.0; NaNO₃, 2.0; K₂HPO₄·3H₂O 1.0; FeSO₄·7H₂O, 0.01; MgSO₄·7H₂O 1.0; K₂CrO₇, 0.1; agar, 20.0; and the initial pH value was adjusted to 7.2 with 1 M NaOH before autoclaving.

The flask fermentation medium was composed of (g/L): sucrose, 30.0; maltose, 5.0; glycerol, 8.0; soy bean, 5.0; NaNO₃, 3.0; monosodium glutamate, 3.0; K₂HPO₄·3H₂O, 1.0; MgSO₄·7H₂O, 1.0; FeSO₄·7H₂O, 0.02; CaCO₃, 3.0; and the initial pH value was adjusted to 7.4 with 1 M NaOH prior to sterilization. All media were sterilized by steam autoclaving at 121°C for 30 min.

**Procedure for screening acarbose producer from soil samples:** The soil samples used in this study were collected from Wuxi, Jiangsu Province. The actinomycetes strains were isolated by the following method. The soil samples were dried at 30°C for 7 days. Each dried soil sample (1.0 g) was suspended in 100 mL of sterile 0.85% NaCl solution in 250 mL flask with 1.0 g glass bead and shook for 5 min. After 10-min-settling, the supernatant (1.0 mL) was added to 9.0 mL of sterile 0.85% NaCl water, after serial dilution to 10⁻⁵ and 10⁻⁶, 0.1 mL of the diluted solution was spread on the plates (three plates for each gradient) and incubated at 28°C for 6-10 days. The single colonies of actinomycetes were then inoculated onto agar plates for further purification. The pure colonies were stored at 4°C in slant agar and in 20% glycerol at -80°C.

The flask fermentation inoculum were prepared by transferring a colony of about 0.5×0.5 cm² size from fresh agar slant to a 250 mL Erlenmeyer flask containing 50 mL of fermentation medium and cultivated at 28°C on a rotary shaker at 200 r/min for 168 h. In the end of the fermentation, the cultures were harvested by centrifugation (6 min, 6000 r/min) in 7 mL centrifuge tubes and the supernatants stored at -20°C until analysis.

**TLC separation:** Acarbose yield in the fermentation broth of actinomycetes was analyzed by TLC. An appropriate amount (5 uL) of each fermentation broth was spotted onto a 10×10 cm silica gel 60 F254 layer. The developing solvent was n-propanol: water (8:2, v/v). Acetone containing 10% (v/v) phosphoric acid, 2% (v/v) aniline and 2% (w/v) diphenylamine was used as color developer. Acarbose on the TLC plate was visualized by the color developer through a fine spray, followed by heating at 110°C for 10 min. To determine the accuracy of the TLC method developed in this study, acarbose in the fermentation broth was also measured by HPLC according to the method as mentioned by Choi and Shin (2003).

**Scanning densitometric conditions:** The scanning densitometer was bought from Shimadzu (CS-9301, Tokyo, Japan). The wavelength scanning range was 370-700 nm, the absorption spectrum was determined by spectrophotometry.

**Standard curve:** A stock solution containing 4.0 mg/mL acarbose was diluted to obtain standard solutions with various concentrations of acarbose ranging from 0.4 to 2.0 mg/mL and then applied to construct the calibration curve for determining acarbose by TLC-scanning analysis.

**Statistical analysis:** All tests and analyses were run in triplicate and results were means of triplicate determinations. Correlation analysis and its significance (p = 0.05) were carried out using SAS (Version 8.0; SAS, Inst., Cary, NC, USA.).

**RESULTS AND DISCUSSION**

**TLC separation acarbose:** A study of the TLC separation of acarbose was performed using a number of solvent systems by changing the ratio of n-propanol and water in the developing solvent. It was found that separation of acarbose could be obtained with the mobile phase containing n-propanol: water (8:2, v/v). The plate, 10×10 cm, was developed by ascending chromatography. In order to achieve good separation, the plate was irrigated two-times with the developing solvent, using an 8-cm path length. Figure 1 revealed the TLC chromatogram of acarbose standard solution. It was achieved after the developed silica gel layer and visualized by the color developer. It was found that the position of acarbose (Rₜ 0.41) was well defined.

**Standard curve of TLC-scanning for acarbose:** In order to determine the wavelength of maximum absorption, wavelength scanning was carried out range from 370 to 700 nm, as shown in Fig. 2. Acarbose on the TLC plate visualized by the color developer and followed by heating (110°C, 10 min) had a wavelength of maximum absorption at 540 nm.
Fig. 1: Thin-layer chromatogram of acarbose (application volume was 5 μL). 1-5: the concentration of acarbose standard solution (mg/mL) was 0.4, 0.8, 1.2, 1.6 and 2.0, respectively. Merck TLC plate, 10×10 cm, was irrigated two-times with 8:2 volume proportions of n-propanol-water, using an 8-cm path length.

Fig. 2: Absorbance spectra of acarbose obtained by TLC-scanning.

Fig. 3: Calibration curve of acarbose standard solution by TLC-scanning.

A calibration curve was constructed for acarbose using the described conditions for TLC separation and scanned at 540 nm. The product peak showed a symmetrical shape with very low baseline noise (data not shown). It could be seen from Fig. 3, the resulting standard curve displayed linearity in the range of 2.0 to 10.0 μg (corresponding to 0.4 to 2.0 mg/mL acarbose in the actinomycetes fermentation broths) of acarbose. Linear regression analysis of the peak area under the curve (y) vs. the concentration of acarbose content (x, mg) gave the equation $y = 108.27x + 432.61$ with an $r^2 = 0.9997$.

Detecting acarbose by TLC-scanning: The solid medium used to isolate actinomycetes was supplemented with K$_2$CrO$_7$ (100 mg/L) to eliminate bacterial contaminants (data not shown), which agreed with Cheng et al. (2008). Eighty soil samples collected from Wuxi, Jiangsu province were screened and 740 actinomycetes strains were isolated from the samples and stored in our lab for further studying their acarbose productivity. After 7 days’ fermentation, the fermentation broths were obtained by centrifugation and used to determine the concentration of acarbose by TLC-scanning.

Although the resolution of TLC was inferior to that of HPLC, some of its properties such as simplicity, economy, easy operation and low consumption of solvents had regenerated interest in TLC (Ohno et al., 2006). TLC identification tests of acarbose were performed using the above developing solvent. The plate was irrigated two-times and good separation of the components in the fermentation broths was achieved. The position of acarbose ($R_f$ 0.41) was well defined and separated from other components present in fermentation broths, as it was shown in Fig. 4.

The developed TLC-scanning technique was checked for its possible application in the screening of high-acarbose producing actinomycetes from 740 actinomycetes strains isolated from soil samples. As shown in Table 1, seven strains of actinomycetes with acarbose yield ranging from 0.45 to 1.83 mg/mL were obtained. The yield was higher than the actinomycetes obtained by Cheng et al. (2008). Therefore, it was...
Table 1: Comparison of the concentration of acarbose determined by TLC-scanning method and HPLC method

<table>
<thead>
<tr>
<th>Strain No</th>
<th>Acarbose content (mg/mL)</th>
<th>HPLC method</th>
<th>TLC-scanning method</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>0.43±0.020</td>
<td>0.45±0.021</td>
<td></td>
</tr>
<tr>
<td>245</td>
<td>0.78±0.035</td>
<td>0.76±0.030</td>
<td></td>
</tr>
<tr>
<td>364</td>
<td>0.53±0.021</td>
<td>0.51±0.020</td>
<td></td>
</tr>
<tr>
<td>382</td>
<td>1.26±0.050</td>
<td>1.23±0.050</td>
<td></td>
</tr>
<tr>
<td>458</td>
<td>1.07±0.044</td>
<td>1.04±0.045</td>
<td></td>
</tr>
<tr>
<td>537</td>
<td>1.86±0.070</td>
<td>1.83±0.087</td>
<td></td>
</tr>
<tr>
<td>642</td>
<td>0.68±0.031</td>
<td>0.67±0.031</td>
<td></td>
</tr>
</tbody>
</table>

Values shown represent means±S.D. of triplicate analysis

Discussion: The TLC-scanning method was effective for the screening of actinomycetes with high acarbose yield.

Determining acarbose in the fermentation broth by HPLC: In order to confirm the accuracy and precision of the established TLC-scanning technique, a comparison was conducted using a HPLC method. As shown in Table 1, the values of acarbose concentration in various fermentation broths measured by TLC-scanning were similar to those determined by the HPLC method. The values of the relative standard deviations between replicate analyses of the acarbose concentrations were found to be within 4.8% (n = 3). These values illustrated that the developed TLC-scanning technique provided high accuracy and precision for the determination of acarbose in the fermentation broths of actinomycetes.

Discussion: TLC method had good recovery, more precision and high sensitivity. Moreover, preparation of the samples was very simple and rapid and derivatization was not needed before chromatography. Therefore, it was widely used in food control, environmental monitoring and pharmaceutical industry (Koobkokkruad et al., 2007).

To ensure the accuracy of this technique, the fermentation medium couldn’t contain starch, because it might be hydrolyzed by the enzymes producing by actinomycetes and form oligosaccharide, such as maltotetraose and isomaltotetraose, which could hamper the separation of acarbose by TLC. Moreover, the TLC plate irrigated two-times was necessary to obtain well separation of acarbose, because acarbose appears to be a tetrasaccharide and it is difficult to separate from tetrasaccharides (Yoon et al., 2003).

TLC separation and scanning (followed by a sensitive chromogenic reagent) possessed several advantages for the preliminary screening of actinomycetes producing acarbose from massive samples:

- Possibility of detection a number of samples per plate
- With no need for complex sample preparation and expensive instrumentation

CONCLUSION

The TLC-scanning method developed in this study was simple and rapid compared to other methods. Therefore it was possible to simply, rapidly and accurately identify actinomycetes capable of producing acarbose by this method. And potentially excellent acarbose producer that could be used in production practice might be discovered. The screening procedure was especially useful in factories and lab with restricted access to more sophisticated and expensive HPLC systems.

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REFERENCES


