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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 ($r^2 = 0.9997$). 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

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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, $K_2HPO_4 \cdot 3H_2O$, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, NaOH, K_2CrO_7, $CaCO_3$, 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; $NaNO_3$, 2.0; $K_2HPO_4 \cdot 3H_2O$ 1.0; $FeSO_4 \cdot 7H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$ 1.0; K_2CrO_7, 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; $NaNO_3$, 3.0; monosodium glutamate, 3.0; $K_2HPO_4 \cdot 3H_2O$, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; $FeSO_4 \cdot 7H_2O$, 0.02; $CaCO_3$, 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 cm^2 size from fresh ager 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 (R_f 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.

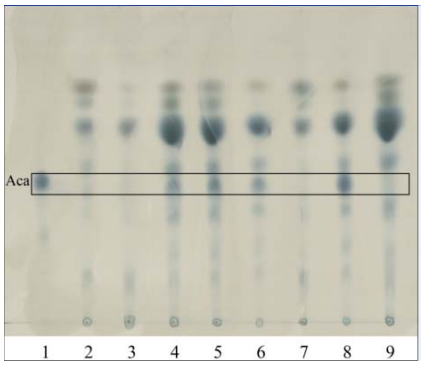

Fig. 1: Thin-layer chromatogram of acarbose (application volume was 5 µL). 1-5: the concentration of acarbose standard solution (mg/mL) was 0.4 0.8, 1.2, 1.6 and 2.0, respectively. Merck TLC plate, 10×10 cm, was irrigated two-times with 8:2 volume proportions of n-propanol-water, using an 8-cm path length

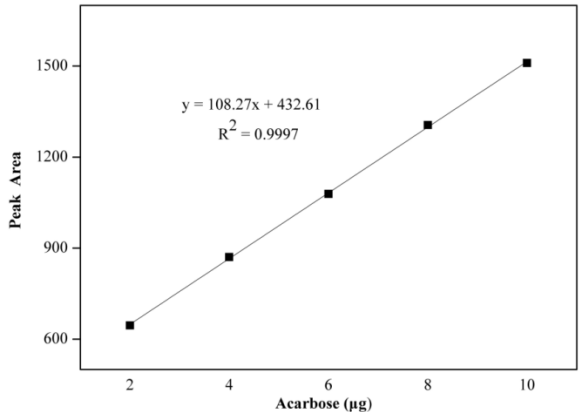

Fig. 2: Absorbance spectra of acarbose obtained by TLC-scanning

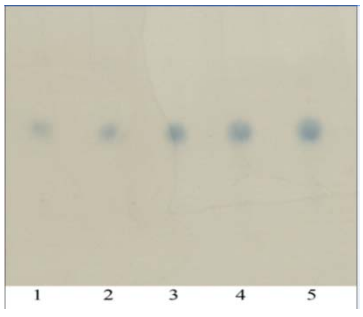

Fig. 3: Calibration curve of acarbose standard solution by TLC-scanning

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. Liner regression analysis of the peak area under the curve (y) vs. the concentration of acarbose content (x, mg) gave the equation $y = 108.27\ x + 432.61$ with an $r^2 = 0.9997$.

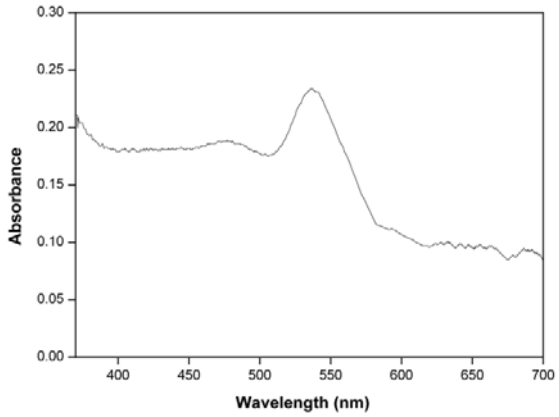

Fig. 4: TLC separation of constituents in the fermentation broths (application volume was 5 µL). Merck TLC plate, 10×10 cm, was irrigated two-times with 8:2 volume proportions of n-propanol-water, using an 8-cm path length. Aca: acarbose; Line 1, acarbose standard; Line 2, 3, 7, 9: actinomycetes fermentation broths without acarbose; Line 4, 5, 6, 8: actinomycetes fermentation broths containing acarbose

Detecting acarbose by TLC-scanning: The solid medium used to isolate actinomycetes was supplemented with K_2CrO_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

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

[a]: Values shown represent means±S.D. of triplicate analysis

obvious that 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
- Reducing the risk of contaminating the stationary phase with successive sample components
- Significantly decreased the time and the cost of reagents relative to HPLC analysis

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.

CONCLUSION

In this study, TLC-scanning, as a simple, sensitive and fast detection method, used to screen acarbose producer was developed. The linearity of the acarbose using this method was good within the range from 2 to 10 μg of acarbose (r^2 = 0.9997). A high-acarbose-producer actinomycete with acarbose yield of 1.83 mg/mL was obtained. The practical application of this work could facilitate the process used for screening strains capable of producing acarbose and screening for higher acarbose producing mutants.

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REFERENCES

Abdelaleem, E.A. and N.S. Abdelwahab, 2013. Stability-indicating TLC-densitometric method for simultaneous determination of paracetamol and chlorzoxazone and their toxic impurities. J. Chromatogr. Sci., 51: 187-191.

Cheng, X., B. Xu, S.J. Wei, X.W. Qiu and G.Q. Tu, 2008. Isolation and identification of a rare actinomycetes strain producing α-glucosidase inhibitor. Food Ferment. Ind., 34: 58-60.

Chiasson, J.L., R.G. Josse, R. Gomis, M. Hanefeld, A. Karasik and M. Laakso, 2002. Acarbose for prevention of type 2 diabetes mellitus: The STOP-NIDDM randomised trial. Lancet, 359: 2072-2077.

Chiasson, J.L., R.G. Josse, R. Gomis, M. Hanefeld, A. Karasik and M. Laakso, 2003. Acarbose treatment and the risk of cardiovascular disease and hypertension in patients with impaired glucose tolerance: The STOP-NIDDM trial. JAMA, 290: 486-494.

Choi, B.T. and C.S. Shin, 2003. Reduced formation of byproduct component c in acarbose fermentation by actinoplane ssp. CKD485-16. Biotechnol. Progr., 19: 1677-1682.

Choi, H., B. Kim, J. Kim and M. Han, 2005. Streptomyces neyagawaensis as a control for the hazardous biomass of Microcystis aeruginosa (*Cyanobacteria*) in eutrophic freshwaters. Biol. Control, 33: 335-343.

Feng, Z.H., Y.S. Wang and Y.G. Zheng, 2011. A new microtiter plate-based screening method for microorganisms producing Alpha-amylase inhibitors. Biotechnol. Bioproc. E., 16: 894-900.

Iwasa, T., H. Yamamoto and M. Shibata, 1970. Studies on validamycins, new antibiotics. I. Streptomyces hygroscopicus var. limoneus nov. var., validamycin-producing organism. J. Antibiot., 23: 595-602.

Kongkiatpaiboon, S., V. Keeratinijakal and W. Gritsanapan, 2013. Simultaneous quantification of stemocurtisine, stemocurtisinol and stemofoline in stemona curtisii (Stemonaceae) by TLC-densitometric method. J. Chromatogr. Sci., 51: 430-435.

Koobkokkruad, T., A. Chochai, C. Kerdmanee and W. De-Eknamkul, 2007. TLC-densitometric analysis of artemisinin for the rapid screening of high-producing plantlets of Artemisia annua L. Phytochem. Anal., 18: 229-234.

Lee, S. and E. Egelkrout, 1998. Biosynthetic studies on the alpha-glucosidase inhibitor acarbose in *Actinoplanes* sp.: Glutamate is the primary source of the nitrogen in acarbose. J. Antibiot., 51: 225-227.

Li, K.T., J. Zhou, S.J. Wei and X. Cheng, 2012. An optimized industrial fermentation processes for acarbose production by Actinoplanes sp. A56. Bioresource. Technol., 118: 580-583.

Miller, G.L, 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Ohno, T., E. Mikami and H. Oka, 2006. Analysis of crude drugs using reversed-phase TLC/scanning densitometry. (II) Identification of ginseng, red ginseng, gentian, Japanese gentian, pueraria root, gardenia fruit, schisandra fruit and ginger. J. Nat. Med., 60: 141-145.

Sahana, A., S. Das, R. Saha, M. Gupta, S. Laskar and D. Das, 2011. Identification and interaction of amino acids with leucine-anthracene reagent by TLC and spectrophotometry: Experimental and theoretical studies. J. Chromatogr. Sci., 49: 652-655.

Schmidt, D., W. Frommer, B. Junge, L. Müller, W. Wingender and E. Truscheit, 1977. α-Glucosidase inhibitors. Naturwissenschaften, 64: 535-536.

Wang, Y., L. Liu, Y. Wang, Y. Xue, Y. Zheng and Y. Shen, 2012. Actinoplanes utahensis ZJB-08196 fed-batch fermentation at elevated osmolality for enhancing acarbose production. Bioresource Technol., 103: 337-342.

Yang, W., J.M. Lu, J.P. Weng, W.P. Jia, L.N. Ji and J.Z. Xiao, 2010. Prevalence of diabetes among men and women in China. New Engl. J. Med., 362: 1090-1101.

Yoon, S.H. and J.F Robyt, 2002. Synthesis of acarbose analogues by transglycosylation reactions of Leuconostoc mesenteroides B-512FMC and B-742CB dextransucrases. Carbohyd. Res., 337: 2427-2435.

Yoon, S.H., R. Mukerjea and J.F. Robyt, 2003. Specificity of yeast (*Saccharomyces cerevisiae*) in removing carbohydrates by fermentation. Carbohyd. Res., 338: 1127-1132.