Separation of Streptothricin Antibiotics from Culture Broth with Colorimetric Determination Using Dipicrylamine

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Our earlier method for the detection and separation of ε-poly-L-lysine using a yellow anionic dye, the dipicrylamine (DPA–) anion, was herein optimized for streptothricin antibiotics (ST), which contains the β-lysine oligopeptides moiety, H–[NH–(CH2)3–CH(NH2)–CH2–CO]n–. We then applied this method to the detection and separation of ST in a commercially available nourseothricin, a mixture of ST species with \( n = 1, 2, 3, \) and 4. The ST species were precipitated with the DPA– anion. The precipitate was found to consist of the salts of the fully protonated ST species, \( \text{ST}^Z (z = n + 1) \), with the DPA– anion. The ST(DPA) precipitate was re-dissolved in acetonitrile. The solution was yellowish, and gave an absorption maximum at around 420 nm. Thus, the equivalent concentration of the ST species referred to the charge numbers of \( \text{ST}^{z+} \) can be determined colorimetrically. By the addition of bis(triphenylphosphoranylidene)ammonium chloride, the ST species could be re-precipitated from the acetonitrile solution as hydrochloride salts. All of the ST species were found in the precipitate with high yields. The method was thus successfully applied to the detection and separation of ST species from the culture broth.

Keywords Separation, colorimetry, streptothricin, lysine oligomer, dipicrylamine

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Introduction

A natural polyamine, ε-poly-L-lysine, exists as the polycationic species under neutral and acidic conditions by the protonation of amino groups, so that it can be detected and separated from the culture broth using anionic reagents, which precipitate the analyte in the polycationic form. In previous work, we developed a method for the colorimetric microtiter plate assay of ε-poly-L-lysine using the dipicrylamine (DPA–) anion, a yellow anionic dye. This method involves precipitating the polycationic species with the DPA– anion and re-dissolving it in acetonitrile (AN). The amount produced in the culture broth can be determined by the yellowness of the AN solution. After a microtiter plate assay, the analyte can be re-precipitated as a hydrochloride salt from the AN solution by the addition of bis(triphenylphosphoranylidene)ammonium chloride. The ST species could be re-precipitated from the acetonitrile solution as hydrochloride salts. All of the ST species were found in the precipitate with high yields. The method was thus successfully applied to the detection and separation of ST species from the culture broth.

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Experimental

Chemicals

Nourseothricin dihydrogen sulfate was obtained from Werner Bioagents. Dipicrylamine sodium salt (NaDPA) was obtained from Tokyo Chemical Industry Co. Ltd. Bis(triphenylphosphoranylidene)ammonium chloride BTPPACl was obtained from Aldrich. 2-Hydroxy-3-morpholinopropanesulfonic acid (MOPSO) was obtained from Dojindo. These were used without further purification. Other chemicals were of reagent grade, and were also used as received.

ST-producing culture broth

Streptothricin was produced according to a previously reported method. A solution containing 10.3% (w/w) sucrose, 3% glucose, 1.5% soytone, 0.1% glycine, 0.1% MgCl₂·6H₂O, and 0.04% CaCl₂·2H₂O was prepared as the culture medium. The pH was adjusted to pH 7.2 with NaOH. Streptomyces lividans TK23 transformant harboring the cosmid vector, pOJ46-SR-cluster7, including the ST biosynthetic gene cluster, was cultivated for 10 days at 28°C with rotary shaking in the culture medium. After centrifugation, the resulting supernatant of the culture broth was used for the detection and separation of ST.

Measurements

The absorbance spectra were recorded by a spectrophotometer (JASCO V-630). In spectrophotometric measurements, the pass length was 1 cm. In the microplate assay, the absorbance was recorded with a microplate reader (Bio-Rad iMark).

The ST compounds were detected and characterized by HPLC/ESI-MS (Bruker Esquire 4000). A Sunniest RP-AQUA column (3.0 μm, 2.1 mm i.d. 100 mm) was used for separation at 25°C. An aqueous solution and an acetonitrile solution were used for mobile phases A and B, respectively. Both solutions contained 0.05% (v/v) n-heptafluorobutyric acid and 0.05% (v/v) formic acid. The A/B mixing ratio was varied as follows: for 0 – 3 min, a 10 – 25% linear gradient of solution B; for 3 – 12 min, a 25 – 35% linear gradient of solution B; for 12 – 16 min, a 35 – 100% linear gradient of solution B; and for 16 – 18 min, a 100% concentration of solution B was used. The flow rate was kept constant at 0.3 mL/min throughout the experiment, and detection was achieved with ESI-MS. Drying gas flow, 10 L/min; drying gas temperature, 365°C; nebulizer pressure, 45 psig.

Microtiter plate assay

The colorimetric ST assay with a multiwell microtiter plate was performed as follows:

1. A 100-μL aliquot of the test solution was transferred into the well.
2. The solution was mixed with 20 μL of the 2 mol/L MOPSO and 1 mol/L NaOH buffer (pH 7.1).
3. The solution was mixed with 50 μL of the 20 mmol/L NaDPA aqueous solution; the mixture was centrifuged, and the supernatant was removed to obtain the ST(DPA) precipitate.
4. The precipitate was mixed with 200 μL of water; the mixture was centrifuged, and the supernatant was removed to wash the precipitate.
5. The precipitate was mixed with 100 μL of AN.

The total concentration of the ST species could be estimated by the yellowness of the AN solution. However, because of the high absorption coefficient of the DPA– anion, 20- and 10-fold dilutions were examined spectrophotometrically (Figs. 3 and 4 below, respectively).

Results and Discussion

According to the data sheet, the nourseothricin sample would consist of ST-F-H₂SO₄, ST-E-1.5H₂SO₄, and ST-D-2H₂SO₄ at 61, 7, and 31% (w/w), respectively. Thus, a 0.28 g/L nourseothricin solution would contain ST-F, -E, and -D at concentrations of ~281, ~25, and ~86 μmol/L, respectively, and the total concentration of ST species may be estimated to be ~1 mEq/L in equivalent concentration referred to the charge numbers in fully the protonated form ST⁺. Curve (a) in Fig. 2 shows a chromatogram of the 0.28 g/L nourseothricin solution. As indicated in the figure, ST-F, -E, and -D were detected. It is noted that the sensitivities differ among the ST species, and that the composition cannot be simply estimated from the peak heights and areas. Although a small peak for ST-C was observed in the chromatogram, the ST species was neglected in the estimation of the equivalent concentration. Even if the sample...
The AN so re above result suggests that the precipitation reaction was absorbance was close to that for the 50 of the AN solution was transferred into another well, and was solution of and spectrophotometrically. As shown in Fig. 4A, the test action:

\[ \text{ST}_m^+ + (\varepsilon - m)\text{H}^+ + z\text{DPA}^- \rightarrow \text{ST(DPA)}_z^- \]  

(1)

A similar precipitation reaction was observed for the colorimetric \( \varepsilon \)-poly-L-lysine assay.\(^4\)

The AN solution was prepared with the test solution at different concentrations of nourseothricin, \( c_{NT} \). A 10-μL portion of the AN solution was transferred into another well, and was mixed with 90 μL of neat AN to prepare the 10-fold dilution. The 10-fold dilution was then examined both colorimetrically and spectrophotometrically.

As shown in Fig. 4A, the test solution of \( c_{NT} = 0 \) gave an almost colorless AN solution, and the yellowness increased remarkably with cNT. The absorbance at 415 nm (because of the limitation of the optical filter) of the AN solution in the well, \( A'_{415} \), increased linearly with \( c_{NT} \) up to 0.5 g/L (Fig. 4B). The regression line is given as

\[ A'_{415} = (2.16 \pm 0.24) (c_{NT} / \text{g L}^{-1}) + (0.00 \pm 0.07), \]  

(2)

with a mean square of errors\(^6\) of 0.0013. The detection limit was calculated to be 3(0.0013)\(^{1/2} \times 2.16 = 0.05 \text{ g/L for nourseothricin.} \)

In the present method, \( A'_{415} \) should be proportional to the concentration of the DPA\(^-\) anion in the AN solution, that is, the equivalent concentration of total ST species in the test solution, \( c_{ST,\text{total}} \) (in mEq/L). Using \( (c_{ST/g} / \text{g L}^{-1}) = 0.28 \times (c_{ST,\text{total}} / \text{mEq L}^{-1}) \), Eq. (2) can be re-written as

\[ A'_{415} = (0.60 \pm 0.07) (c_{ST,\text{total}} / \text{mEq L}^{-1}) + (0.00 \pm 0.07). \]  

(2')

Separation of ST after the colorimetric determination

The ST species in the AN solution can be re-precipitated as hydrochloride salts ST(Cl)\(^-\), by the addition of BTPPACl, according to the following reaction:

\[ \text{ST(DPA)}, + z\text{BTPPADPA} \rightarrow \text{ST(Cl)}, + z\text{BTPPADPA}. \]  

(3)

The AN solution was prepared with the 0.28 g/L nourseothricin test solution (100μL), and was treated according to procedures (6) and (7) above. The resulting precipitate was mixed with 100 μL of water. After centrifugation, no sediment was found in the mixture, indicating that the precipitate was completely dissolved into water. Curve (b) in Fig. 2 shows a chromatogram of the aqueous solution. Elution peaks for ST-F, -E, -D, and -C were observed remarkably. From the peak areas, the yields for ST-F, -E, -D, and -C were calculated to be 69, 73, 90, and 91%, respectively, suggesting that the yield would decrease slightly with decreasing \( n \).

In a previous bioengineering study,\(^7\) the ST species were separated and purified from the culture broth by using the tetraphenylborate anion. This method also involves precipitating ST\(^+\) with the anionic reagent and re-precipitating the ST species as hydrochloride salts.\(^3\) In this case, the yields for ST-F and -E were poor. Also, the tetraphenylborate anion precipitates NH\(_4\)\(^+\) and K\(^+\) cations,\(^11,12\) which are often added into the culture medium. On the other hand, the DPA\(^-\) anion did not precipitate these cations, even at the 100 mM/L level. Furthermore, unlike ST\(^+\) salts with the tetraphenylborate and 12-molybdosilicate\(^1\) anions, ST(DPA)\(^-\) is soluble in AN. This may be due to the plane structure of the DPA\(^-\) anion.\(^13\) Thus, the DPA\(^-\) anion can be considered to be a superior analytical reagent for ST.

Other polyamines

In our previous paper,\(^1\) we showed that the present colorimetric method can be applied to the assay for large-size polyamines, such as \( \alpha \)-poly-L-lysine hydrobromide (molecular weight, \( M_e = 15000 - 30000 \)), glycol chitosan (\( n > 400 \)), poly(allylamine hydrochloride) (\( M_e = 15000 \)), and branched polyethyleneimine (\( M_e = 750000 \)). The hydrophobicity of the DPA\(^-\) anion\(^4\) is likely to be an important factor for the precipitation of the polycations. Previous studies showed a significant interaction between the polycationic ST and \( \varepsilon \)-poly-L-lysine and a hydrophobic anion in a non-aqueous phase.\(^8,15\)

In this study, we preliminarily investigated the applicability of our method to the assay of natural polyamines, which would possess a few or several positive charges. Putrescine, spermidine, and spermine would exist as di-, tri-, and tetravalent cationic species, respectively, in fully protonated form. The 1 mEq/L solution of spermidine and spermine gave yellowish precipitates

Fig. 4 (A) 10-Fold dilution of AN solutions given by test solutions with different concentrations of nourseothricin, \( c_{NT} = 0, 0.1, 0.2, 0.3, 0.4, \) and 0.5 g/L. (B) Plot of the absorbance at 415 nm for 10-fold dilution in microplate, \( A'_{415} \), against \( c_{NT} \).
with the DPA anion, but the putrescine solution gave no precipitate. Streptomycin, kanamycin, tobramycin, and neomycin would exist as tri-, tetra-, penta-, and hexavalent cationic species, respectively. The 1 mEq/L solutions of streptomycin and kanamycin gave no precipitate, while those of tobramycin and neomycin gave a precipitate. Although chitosan oligosaccharide would possess several positive charges, no precipitate was formed significantly with the DPA anion.

Since the DPA anion precipitates even ST-F, the interaction of the anion with the protonated lysine residue may be stronger than that with other ammonia groups. However, monomeric lysine, which would exist as a zwitter ionic species, gave no precipitate. Thus, the method may also be applied to the assay of other compounds containing lysine oligomer residues.

Separation from culture broth

The culture medium gave no precipitate with the DPA anion, indicating that the cation components, such as Mg$^{2+}$ and Ca$^{2+}$, would not interfere with the present ST assay. S. lividans TK23 transformant harboring the cosmid vector, pOJ446-SR-cluster7, including the ST biosynthetic gene cluster was cultivated to obtain an ST-producing culture broth. Curve (a) in Fig. 5 shows a chromatogram of the culture broth. The production of ST-F, -E, -D, and -C was confirmed by observing their well-developed elution peaks. It is noted that the composition was different from that of nourseothricin. Also, a significant background signal and impurity peaks were observed in the chromatogram.

The present colorimetric plate assay was performed with the culture broth. The resulting AN solution was yellowish, and gave $A_{415} = 1.0 \pm 0.12$. Using Eq. (2′), the produced amount was determined to be $c_{\text{ST, total}} = 1.8 \pm 0.3$ mEq/L. Then, the ST-species were isolated from the AN solution, and were re-dissolved into 100 μL of water. Curve (b) in Fig. 5 shows a chromatogram of the aqueous solution. All of the ST species were detected, and the background signal and impurity peaks were no longer observed significantly. After a correction for the background signal in curve (a), the yields for ST-F, -E, -D, and -C were estimated to be 70, 75, 79, and 82%, respectively.

Thus, the ST species could be separated and purified successfully from the culture broth. Also, the results in chromatographic analysis indicate again the selectivity of the colorimetric determination.

The above results indicate that the present microtiter plate assay was able to detect the ST species in the culture broth. After the assay, the ST species could be isolated to obtain purified products with high yields. The present determination and separation method is rapid and easy to carry out, and would be useful for studies on the production of ST and its analogues. Such a bioengineering study is underway.

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