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Analysis of N_2-(2-carboxyethyl)-L-arginine, an intermediate of clavulanic acid, by liquid chromatography method using benzoin as a fluorogenic reagent

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Abstract

Clavulanic acid (CA) is a commercially important broad-spectrum β-lactamase inhibitor that was produced by *Streptomyces clavuligerus*. The first step in the biosynthesis of CA is the condensation of two primary metabolites, D-glyceraldehyde 3-phosphate and L-arginine, to give N_2-(2-carboxyethyl)-L- arginine (CEA). Analysis of CEA in the fermentation broth of *Streptomyces clavuligerus* RFL35 is investigated by using benzoin as a fluorogenic reagent. The method is based on reverse-phase high performance liquid chromatography (HPLC) following pre-column derivatization with benzoin and fluorescence detection. CEA appears in chromatogram in a retention time of 4.7 min. This finding may help to enhance CA production by optimizing the fermentation medium and genetic manipulation of *Streptomyces clavuligerus*. It may also facilitate the structural and mechanistic studies on N_2-(2-carboxyethyl)-L-arginine synthase that catalyzes an unusual N-C bond forming reaction.

Keywords: Actinomycetes, Benzoin, Clavulanic acid, N_2-(2-carboxyethyl)-L-arginine, *Streptomyces clavuligerus* RFL35.

Introduction

N_2-(2-carboxyethyl)-L-arginine synthase (CEAS), an unusual thiamin diphosphate (ThDP)-dependent enzyme, catalyses the first step in the biosynthesis of the β-lactamase inhibitor clavulanic acid in *Streptomyces clavuligerus*. The first step in the biosynthesis of clavulanic acid is the condensation of two primary metabolites, D-glyceraldehyde 3-phosphate and L-arginine, to give N_2-(2-carboxyethyl)-L- arginine (CEA) (Caines *et al*., 2004).

Detection of intermediates is very useful in mutagenesis and genetic engineering studies. It can be used to find specific high-producer strains. Analysis of CEA as a guanidino compound is quite difficult due to its poor detectability owing to the absence of strong chromophore and fluorophore. A sensitive fluorimetric method for determination of monosubstituted guanidine compounds based on their reaction with benzoin is introduced (Ohkura and Kai, 1979). This method has shown good precision and sensitivity, and requires only minimal sample handling. It is simple and perfect for monosubstituted guanidine compounds (Kai *et al*., 1981, 1984, 1985). The benzoin reaction does not give fluorescent derivatives of biological substances having no guanidine moiety in the molecule (Kai *et al*., 1984). The excitation and emission spectra of the fluorescences from monosubstituted guanidine compounds and arginine are very similar in shape and maxima. Therefore, they are not characteristic of individual compounds and the compounds with one guanidino group in their molecule give nearly equal fluorescence

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intensities at equimolar concentrations (Ohkura *et al*. 1979). However, this method had not been used for the assay of CEA previously. The aim of the present study is the detection of CEA in the fermentation broth of *Streptomyces clavuligerus*.

Materials and Methods

Bacterial Strains, Culture Media and Method

Streptomyces clavuligerus DSM 738 (from Deutsche Sammlung von Mikroorganismen und Zellkulturen) and *Streptomyces clavuligerus* RFL35, a mutant that is generated by inactivating the β-lactam synthetase's encoding gene, resulting in the accumulation of CEA (Bachmann *et al*. 1998). The later strain was obtained as a gift from Professor Townsend (Johns Hopkins University, USA). Both of *S. clavuligerus* strains were inoculated on sporulation medium (Shirling and Gottlieb, 1966) [(g/l): glucose 4, malt 10, yeast extract 4, $CaCO_3$ 2, agar 20] and incubated at 28 ^oC for 14 days. One ml of spore suspension (ca. 10^7-10^8spores per ml) was inoculated in 1000 ml Erlenmeyer flasks containing 250 ml of seed medium (Cole, (77) ([(g/l): peptone 10 g, glycerol 20 g, malt 10, pH 7.0±0] and incubated at 28°C on a rotary shaker at 220 rpm for 20-22 h. Then, the seed culture was inoculated at 5% (v/v) into 250 ml Erlenmeyer flasks containing 50 ml fermentation medium and incubated at 28°C for 96 h at 220 rpm. The cultures of *S. clavuligerus* RFL35 and *S. clavuligerus* DSM738 were grown in starch asparagine (SA) fermentation medium (Paradkar *et al*. 1995) and soy bean flour (soy) fermentation medium (Hamedi *et al*. 2007).

Sakaguchi Analysis

Guanidino–containing compounds were detected in cultures by Sakaguchi analysis (Ceriotti and Spandrio, 1957). The fermentation broth was centrifuged for 30 min, 2600 g, 4˚C, to remove the biomass and insoluble medium ingredients then analyzed by Sakaguchi analysis.

Liquid Chromatographic Analysis of N_2-(2-Carboxyethyl)-L-Arginine

The fermentation broth was centrifuged for 30 min, 2600 g, 4˚C, to remove the biomass and insoluble medium ingredients. Then, the supernatant was separated and the pH adjusted to 2.8±0.1 by drop-wise addition of HCl (5N). It was centrifuged for 30 min, 2600 g, 4˚C, to precipitate proteins. The supernatant was separated and its pH was adjusted to 6.5-7 by drop-wise addition of KOH (5M). The diluted samples were derivatized as described by Kai *et al*. (1983). The solutions needed for the derivatization reaction of CEA and arginine by benzoin were prepared as described by Kai & *et al* (1983). The high performance liquid chromatography (HPLC) system consisted of a Wellchrom K-1001 HPLC pump and a Knauer RF-10AXL HPLC fluorescence spectrophotometer detector with a xenon lamp. The pH-resistant Shodex C18 column (D18-613, Showa Denko k. k., Japan). The mobile phase consisted of methanol-water-0.5 M Tris-hydrochloric acid buffer (pH 8.5) (55:30:15) at total flow rate 0.8 ml/min. Fluorescence detection at λ_{em}= 425 nm with λ_{ex}= 325 nm was used. The column temperature was 27°C and sample injection volume was 100 µl.

Assay of Clavulanic Acid

Concentration of clavulanic acid in the fermentation broth was determined by HPLC, as described by Foulstone and Reading (1982). This method is based on measuring the absorbance of imidazole-derived products of clavulanic acid at 311 nm. A C18 (250×4.6 mm, Hichrom, UK) column was used. The mobile phase consisted of methanol (30%) and 50 mM phosphate buffer (70%), total flow rate 0.5 ml/min. The column temperature was 27°C and sample injection volume was 20 µl. A potassium clavulanate standard was provided by Kosar Pharmaceutical Co., Tehran, Iran.

Results

Concentration of Metabolite(S) Containing a Guanidine Functional Group in Fermentation Broth

SA and soy fermentation broths of *S. clavuligerus* RFL35 and *S. clavuligerus* DSM 738 were analyzed for detection of guanidine-containing compound(s) by the Sakaguchi color reaction and the result was shown in the Fig. 1.

As seen, no significant guanidine containing compound(s) was detected in the fermentation broth of *S. clavuligerus* DSM 738. But, the concentration of these compounds is detectable in the culture filtrate of *S. clavuligerus* RFL35. Also, the concentration of metabolite(s) containing a guanidine functional group in soy fermentation broth of *S.clavuligerus* RFL35 was 2.42 times more than that of SA fermentation broth.

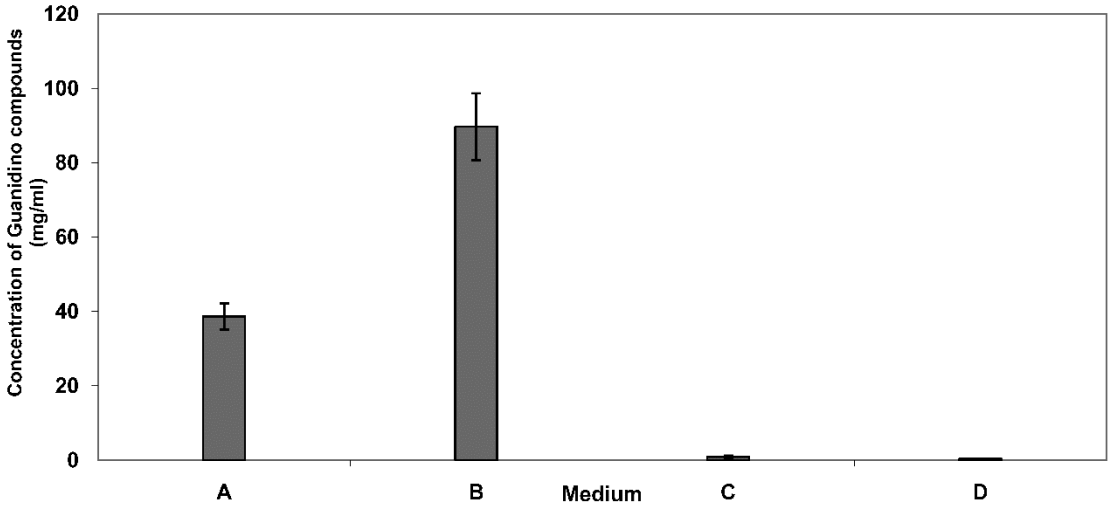

Figure 1. Accumulation of the metabolite(s) containing a guanidine functional group in the fermentation broth of *S. clavuligerus*. A, C: SA fermentation broth, B, D: Soy fermentation broth. A, B: *S. clavuligerus* RFL35; C, D: *S. clavuligerus* DSM738.

Detection of Blank and Arginine's Peaks in Chromatogram

To find the accompanying peaks in the analysis of CEA, deionized water and arginine were analyzed by the derivatization procedure used. They were analyzed by the fluorimetric liquid chromatography method using benzoin and the results are shown in Fig. 2. The retention times of the blank and arginine were seen at 3.2 and 16 minutes, respectively.

Comparison of Chromatograms in Soy Medium Fermentation Broth of *S. clavuligerus*

Soy fermentation broths of *S. clavuligerus* RFL35 and *S. clavuligerus* DSM 738 were used for the analysis of guanidine-containing compound(s) by fluorimetric liquid chromatography method using benzoin, and the results are shown in Fig. 3. A large quantity of metabolite containing monosubstituted guanidine compound was detected in the fermentation broth of *S. clavuligerus* RFL35 with a retention time of 4.7 min (Fig. 3a), but no significant guanidine containing compound was detected in the fermentation broth of *S. clavuligerus* DSM 738 in this retention time (4.7 min) (Fig. 3b).

Chromatograms of guanidine-containing compound(s) in SA medium fermentation broth of *S. clavuligerus*

SA fermentation broths of *S. clavuligerus* RFL35 and *S. clavuligerus* DSM 738 were used for the

analysis of guanidine-containing compound(s) by fluorimetric liquid chromatography method using benzoin, and the results are shown in Fig. 4. A large quantity of metabolite containing monosubstituted guanidine compound was detected in the fermentation broth of *S. clavuligerus* RFL35 with a retention time of 4.7 min (Fig. 4a), but no significant guanidine containing compound was detected in the fermentation broth of *S. clavuligerus* DSM 738 in this retention time (4.7 min) (Fig. 4b).

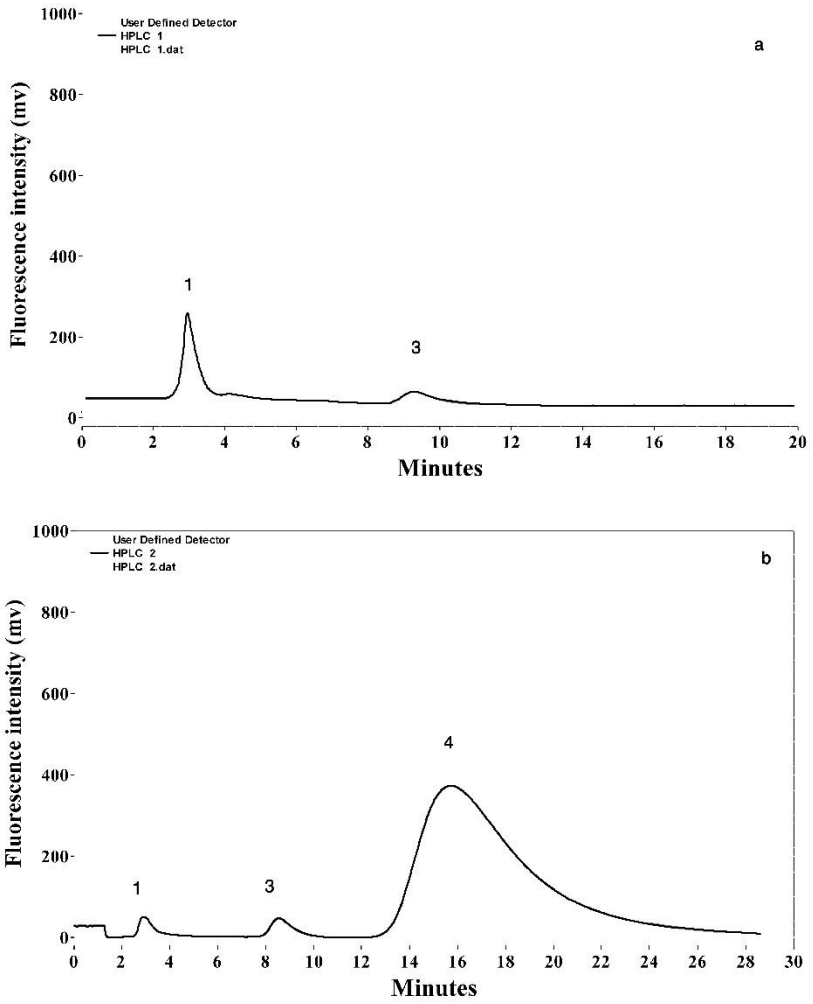

Figure 2. Detection of blank and arginine's peaks in medium fermentation broth. a: deionized water, b: arginine. Peaks: 1, blank. 3, blank. 4, arginine.

Chromatograms of Guanidine-Containing Compound(S) of *S. clavuligerus* RFL35 In Soy And SA Fermentation Media

When chromatograms of soy and SA fermentation broths of *S. clavuligerus* RFL35 were compared, it was seen that the concentration of monosubstituted guanidine compound in retention time 4.7 min in soy medium was 2.12 times more than that of SA medium fermentation broth of *S. clavuligerus* RFL35 (Fig. 3a and Fig. 4a).

Clavulanic Acid Production by *S. clavuligerus* DSM 738 in Soy and SA Fermentation Media

The concentration of clavulanic acid by *S. clavuligerus* DSM 738 in the soy medium was two times more than that in SA medium (Fig. 5).

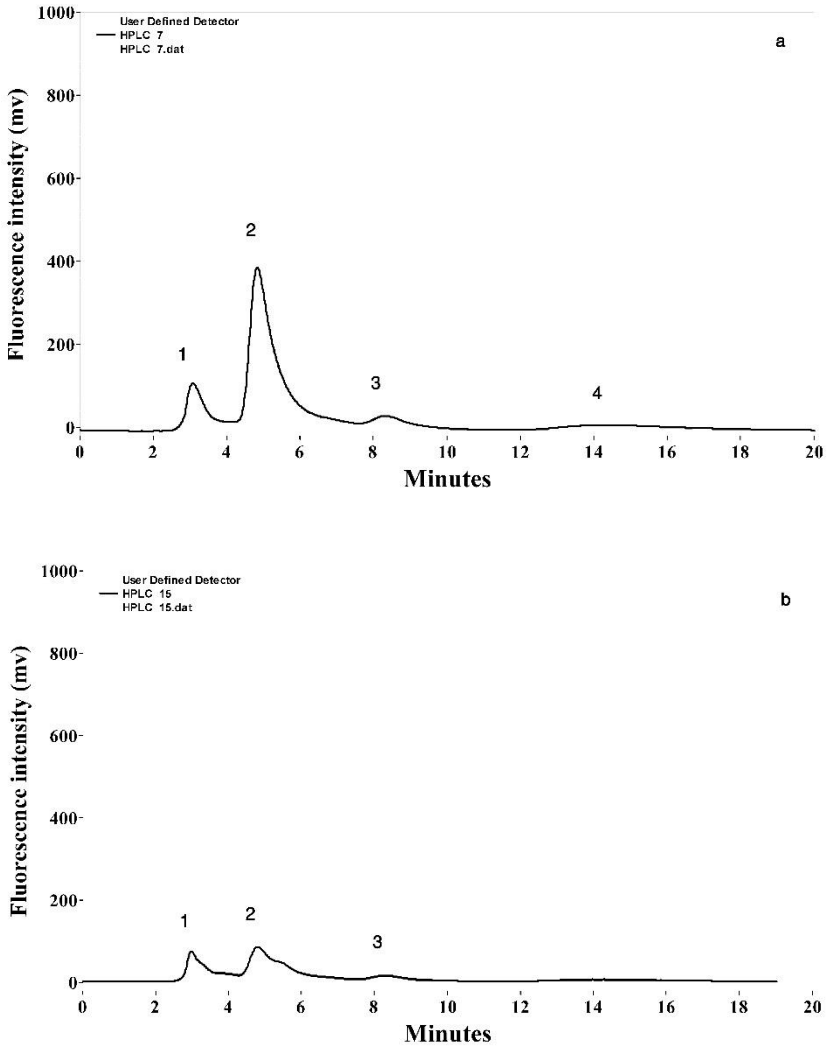

Figure 3. The comparison of chromatograms in Soy medium fermentation broth. a: The culture of *S. clavuligerus* RFL35, Peaks: 1, blank: 2, monosubstituted guanidine compound (CEA): 3, blank. 4, arginine. b: The culture of *S. clavuligerus* DSM 738. Peaks: 1, blank. 2, monosubstituted guanidine compound. 3, blank.

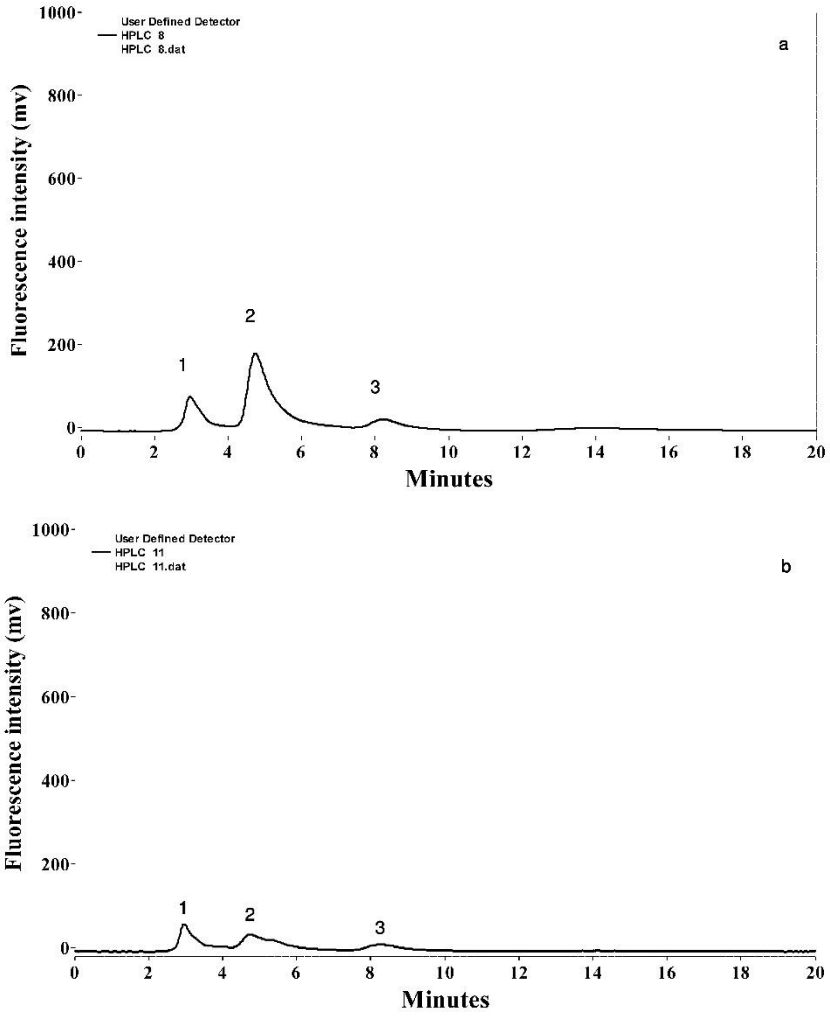

Figure 4. The comparison of the chromatograms in SA medium fermentation broth. a: The cultures of *S. clavuligerus* RFL35, Peaks: 1, blank: 2, monosubstituted guanidine compound (CEA): 3, blank. b: The cultures of *S.clavuligerus* DSM 738. Peaks: 1, blank. 2, monosubstituted guanidine compound. 3, blank.

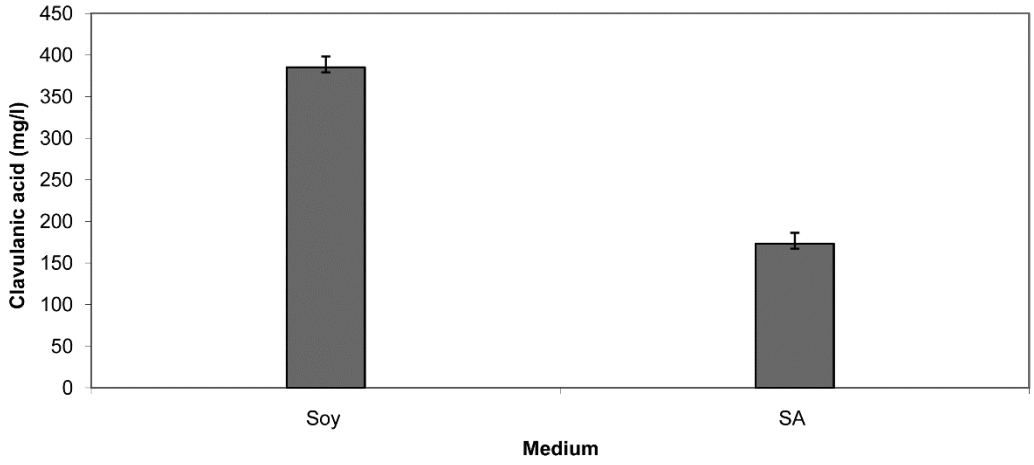

Figure 5. Clavulanic acid production by *S. clavuligerus* DSM 738 in soybean (Soy) and Starch asparagines (SA) fermentation media.

Discussion

N_2-(2-carboxyethyl)-L-arginine is a monosubstituted guanidine compound that is synthesized by N_2-(2-carboxyethyl)-L-arginine synthase (*Orf* 2) in *S. clavuligerus* (Foulstone and Reading, 1982). This compound is the substrate of β-lactam synthetase (*Orf* 3) to produce deoxyguanidinioproclavaminic acid (Bachmann *et al*., 1998). It is obvious that no CEA could be accumulated in the fermentation broth of the wild strain of *S. clavuligerus* (DSM 738), because it was consumed in the biosynthetic pathway of CA. However, it was accumulated in the fermentation broth of *S. clavuligerus* RFL35, a mutant that was generated by inactivating β-lactam synthetase's encoding gene, resulting in the accumulation of CEA (Bachmann *et al*., 1998).

By comparing Figs (2-4), it can be concluded that the peak with retention time 4.7 min belongs to CEA. Benzoin reaction is a selective method for monosubstituted guanidine compounds and no fluorescent derivatives of biological substances without guanidine moiety can be seen (Kai *et al*., 1981). Furthermore, CEA has two carbonyl groups with pH 8.5 (mobile phase) and therefore, has high polarity. It seems the peak of CEA appeared at the beginning of the chromatogram. A similar result was reported for guanidinosuccinic acid (Kai *et al*., 1981).

S. clavuligerus RFL35 produced more metabolite containing monosubstituted guanidine compound (with retention time 4.7 min) in the soy medium than in the SA medium. *S. clavuligerus* DSM 738 produced more clavulanic acid in the soy fermentation medium (Fig. 5). It was showed that the concentration of clavulanic acid in soy medium was more than that in SA medium (Jensen *et al*. 2000). It can be concluded that greater concentration of CEA resulted in greater concentration of CA. Liquid chromatography/mass spectrometry (LC/MS) analysis had been used to analyze N_2-(2-carboxyethyl)-L- arginine in structural and mechanistic studies on N_2- (2-carboxyethyl)-L-arginine synthase, an unusual thiamin diphosphate (ThDP)- dependent enzyme, previously (Caines *et al*., 2009). Benzoin has been shown as a useful reagent for derivatization of CEA needless of mass spectrometer or the extraction of CEA. The analysis of guanidino compounds with benzoin is simple and prefect, but the C18 column degrade in high pH of the mobile phase. In this paper Shodex C18 column having large pH stability range of 1-12 was used for isolation of CEA. The reagent exhibited good reactivity and selectivity for CEA. The method proposed for CEA analysis could be useful to find high-CEA-producer mutants of *Streptomyces clavuligerus* and study of the mechanism and activity of CEA synthase in *Streptomyces clavuligerus.*

Acknowledgments

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