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Short Communication

Application of ligand-exchange chromatography to the assay of L-alanine from DL-aspartic acid by *Pseudomonas dacunhae*

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ABSTRACT

A direct chiral ligand-exchange chromatographic method was developed to monitor L-alanine production by fermentation. A mobile phase containing aqueous 0.25 m*M* Zn^{2+} solution is utilized to separate amino acids in the fermentation medium. The detection limit for L-alanine is 0.5 ppm and the analysis time for one sample is about 8 min. As sample preparation is simple and the matrix effects are minimal, the assay is fast and convenient. The results indicate that the method has potential for the analysis of complicated fermentation media.

INTRODUCTION

Although the amino acid analyser is routinely used for the determination of amino acids in various biological fluids, ligand-exchange chromatographic methods, pioneered by Davankov and co-workers [1,2], have been developed for analysing amino acid enantiomers with a chiral stationary phase [3,4] and the high selectivity and specificity of ligand-exchange chromatography has led to many applications. Recently, the resolution of DL-amino acids on MCI GEL CRS10W and CRS10WD columns based on the ligand-exchange reactions between the copper complex of an optically active ligand adsorbed on the column matrix and the amino acid to be separated has been reported [5]. The enantiomers of amino acids were determined with the aid of ligand-exchange chromatography in chiral complex-containing mobile phases by Duchateau *et al.* [6]. Fukuhara and Yuasa [7] demonstrated that fourteen DL-amino acids can be resolved by using a chiral cyanocobalamin-coated ligand-exchange column with a mobile phase containing copper complexes of ADP, NAD or FAD as a chiral additive [8]. Racemic α-alkyl-α-amino acids can be analysed by ligand-exchange chromatography with L-3-carboxy-1,2,3,4-tetrahydroquinoline as the bonded chiral selector [9]. Moreover, even α-CF_3AA enantiomers can be separat-

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ed on L-proline and L-hydroxyproline sorbents with Cu^{2+} ions in the aqueous mobile phase [10]. However, the application of ligand-exchange chromatography to the analysis of fermentation media containing amino acids has received little attention.

In our laboratory, the direct ligand-exchange separation of α-amino acid enantiomers without any derivatization has been found useful for monitoring the amino acid fermentation system [11]. The developed method used a commercially available column with silica gel modified with a chiral aliphatic amino acid–Cu^{2+} complex as the stationary phase and an achiral aqueous solution containing Zn^{2+} ion as the mobile phase. The separation of L-alanine from other amino acids in a complicated fermentation medium can thus characterize the applicability of ligand-exchange chromatography.

EXPERIMENTAL

Materials

DL-Aspartic acid, D-aspartic acid, L-aspartic acid and L-alanine were purchased from Sigma (St. Louis, MO, USA). $ZnSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, K_2HPO_4, extract of dried meat and sodium L-glutamate were obtained from Merck (Darmstadt, Germany). The high-performance liquid chromatographic (HPLC) column (25 cm × 4.6 mm I.D.) was packed with a chemically bonded aliphatic amino acid–Cu^{2+} complex on silica gel (5 μm) (Tosoh, Tokyo, Japan). Water was deionized and filtered before use.

Instrumentation

The HPLC system consisted of a Shimadzu (Kyoto, Japan) LC-9A dual-piston solvent-delivery module with a high-sensitivity filter unit, a Rheodyne (Cotati, CA, USA) Model 7125 injection valve with a 20-μl sampling loop, a Shimadzu Chromatopac CR-6A data processor and a Shimadzu Model SPD-6A variable-wavelength UV detector. The detection wavelength was at 254 nm and the column operating temperature was at 26°C. The isocratic elution rate of the mobile phase was at 1.0 ml/min.

Fermentation

Pseudomonas dacunhae (CCRC 12623; Hsinchu, Taiwan) was incubated with the following growth medium [11]: 8 g of sodium L-glutamate, 1.25 g of extract of dried meat, 0.125 g of KH_2PO_4, 0.81 g of K_2HPO_4 and 0.025 g of $MgSO_4 \cdot 7H_2O$ in 250 ml of water. The bacteria were grown in this medium for 4 days, then about 50 ml of the medium were transferred into 100 ml of fermentation medium, consisting of 0.125 g of KH_2PO_4, 0.81 g of K_2HPO_4, 0.025 g of $MgSO_4 \cdot 7H_2O$ and 2.5 g of DL-aspartic acid per 250 ml of water. The inoculated fermentation medium was incubated in an orbital incubator–shaker (Model 702, Hotech, Taipei, Taiwan) for 7 days at 30°C with a shaker speed 120 rpm. No foaming was observed during fermentation.

Sample preparation

Stock standard amino acid solutions were prepared by measuring suitable amounts of analytical-reagent grade amino acids and dissolving them in water, and successive dilutions were utilized to obtain lower concentrations. Mild heating was

needed to dissolve both D- and L-aspartic acid. A 0.20-μm microporous membrane filter (Gelman Science, Ann Arbor, MI, USA) was used to filter off the bacterial cells from the fermentation medium. The filtered medium was diluted with water to a concentration range suitable for analysis.

RESULTS AND DISCUSSION

Qualitative separation

L-Alanine produced in the fermentation medium can be separated from DL-aspartic acid by using aqueous eluents containing Zn^{2+} ion or Cu^{2+} ion or their mixture. As the formation constant of $Cu(NH_3)_4^{2+}$ is larger than that of $Zn(NH_3)_4^{2+}$, the free amino acid–Zn^{2+} complexes should be less stable than the free amino acid–Cu^{2+} complexes. Cu^{2+} (with a d^9 electron configuration) uses a dsp^2 hybrid orbital to form stable square-planar complexes with amino acids, whereas Zn^{2+} (with a d^{10} electron configuration) usually forms tetrahedral complexes via an sp^3 hybrid orbital. Therefore, the separation of amino acids by different metal ions is mainly based on the different stability of the complexes formed. The structure of the amino acid is a determining factor in the separation.

Fig. 1 shows that the best resolution between L-alanine and DL-aspartic acid is represented by chromatogram A, obtained by elution with an aqueous mobile containing 0.25 mM Zn^{2+}. Also, only the aqueous mobile phase containing Zn^{2+} can resolve the L-glutamic acid and DL-aspartic acid. The separation of L-glutamic acid and DL-aspartic acid is not successful with the aqueous mobile phase containing Cu^{2+}.

The experiment was repeated many times with an aqueous mobile phase con-

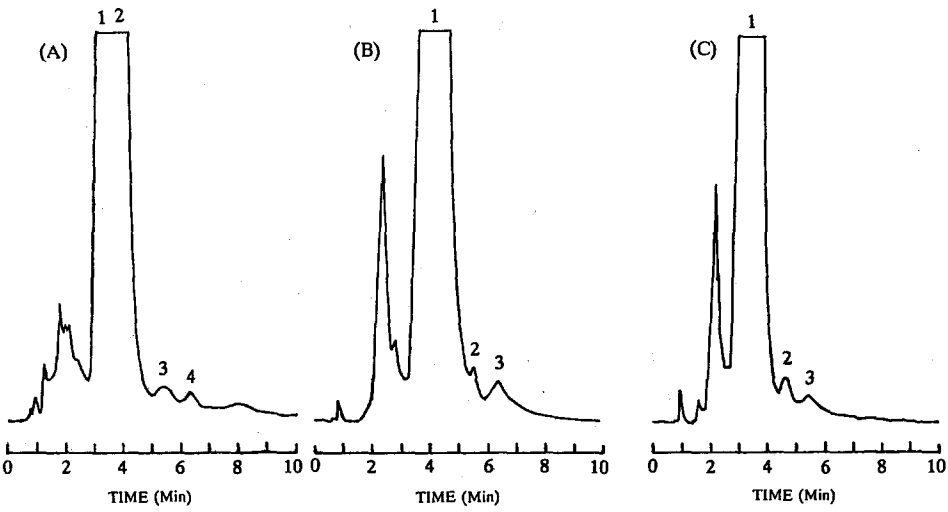

Fig. 1. Effect of mobile phase on the separation of the 10-fold diluted fermentation medium. (A) Mobile phase: 0.25 mM Zn^{2+}; peaks: 1 = L-glutamic acid; 2 = DL-aspartic acid; 3 = L-alanine; 4 = unknown. (B) Mobile phase: 0.125 mM Cu^{2+}; peaks: 1 = L-glutamic acid and DL-aspartic acid; 2 = L-alanine; 3 = unknown. (C) Mobile phase: 0.125 mM Cu^{2+}–0.25 mM Zn^{2+} (45:55); peaks: 1 = L-glutamic acid and DL-aspartic acid; 2 = L-alanine; 3 = unknown.

taining Zn^{2+} and demonstrated good detection sensitivity at UV wavelength of 254 nm and good stability of the column. There is no problem with replacement of Cu^{2+} ions bonded on the silica gel surface by a Cu–Zn exchange equilibrium. Thus, the eluent selected at present stage is 0.25 m*M* aqueous Zn^{2+} solution [12]. Verification of peaks was achieved by adding standard amino acid solutions to the medium. A very small peak is produced by the medium that cannot be identified.

After fermentation the pH of the fermentation medium was 8.42 and with 100-fold dilution it was 8.38. DL-Aspartic acid enantiomers in the medium cannot be resolved under basic conditions, as shown in Figs. 1 and 2A. The fermentation medi-

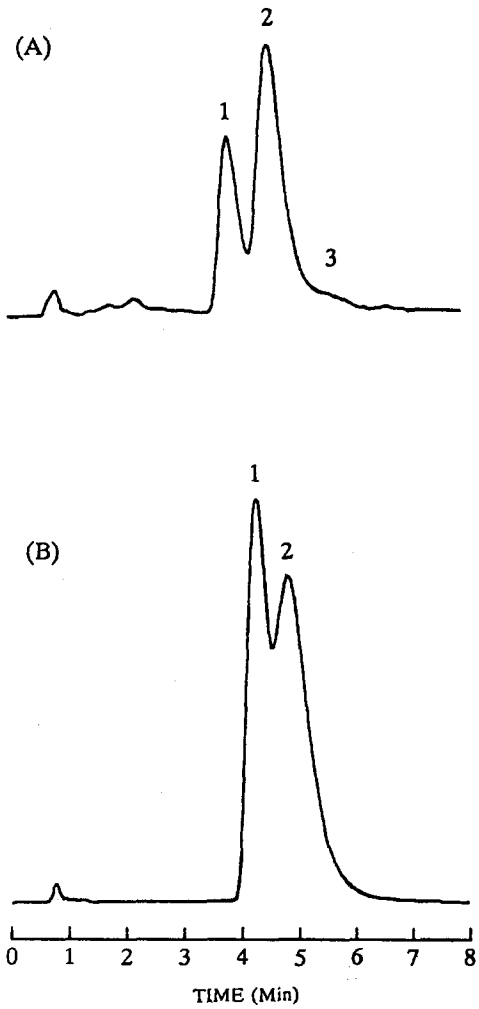

Fig. 2. Effect of pH on the separation of DL-aspartic acid with 0.25 m*M* Zn^{2+} aqueous solution as mobile phase. (A) Medium: sodium L-glutamate, DL-aspartic acid, extract of dried meat, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$; 100-fold dilution; pH 8.38; peaks: 1 = L-glutamic acid; 2 = DL-aspartic acid; 3 = L-alanine. (B) Medium: DL-aspartic acid, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$; 100-fold dilution; pH 3.74; peaks: 1 = D-aspartic acid; 2 = L-aspartic acid.

um without DL-aspartic acid and without a fermentation process occurring had a pH of 7.11. The pH of the medium containing DL-aspartic acid, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4–K_2HPO_4 buffer measured before fermentation was 3.37. After 100-fold dilution, the former medium had a pH 3.74, as shown in Fig. 2B. DL-Aspartic acid enantiomers can be separated slightly under acidic conditions with 0.25 m*M* Zn^{2+} aqueous mobile phase. We also found that DL-aspartic acid enantiomers dissolved in water alone were better separated under the same chromatographic conditions [12]. Therefore, the simultaneous determination of D-aspartic acid, L-aspartic acid and L-alanine is possible under acidic conditions. However, substances present in or the ionic strength of the fermentation medium may have crucial effects on the separation of L-alanine and DL-aspartic acid enantiomers. We do not intend to consider the detailed separation mechanism of DL-amino acid enantiomers here, but we believe that molecular distortion of amino acids toward different irregular tetrahedral structures may play an important role in their separation.

Quantitation

The amount of L-alanine produced by fermentation was determined by using external calibration. Experimental data for D-aspartic acid, L-aspartic acid and L-alanine were fitted to the linear relationship $y = ax + b$ by non-linear regression (NLR) analysis. The regression procedure is based on the Marquart algorithm which utilizes an interpolation technique to combine the Gauss–Newton and steepest descent search method [13]. The slope of the calibration graph for L-alanine was 3423.0 ± 87.74 and the intercept was −4407.2 ± 1971 at the 95% confidence level. The precision for L-alanine determination was usually about 2 within the concentration range 50–5 ppm. The amount of L-alanine produced by the fermentation was about 104.2 ppm. The accuracy of this value is slightly affected by tailing, as shown in Fig. 1, and a correction should be made by analysing standard material. However, here we only want to know whether the developed ligand-exchange chromatographic procedure is adequate for monitoring an amino acid fermentation system or not. The yield of L-alanine in the medium is low, indicating a low activity of the enzyme L-aspartate β-decarboxylase. Shibatani *et al.* [14] reported that the optimum pH for L-aspartate β-decarboxylase was 5.3 and the maximum decarboxylation temperature was 47°C. Utilizing the whole cell of *Pseudomonas dacunhae* for fermentation, we prefer to keep the pH at 6.2 and maintain a temperature at 37°C [10,11]. Nevertheless, the detection limit for L-alanine is 0.5 ppm with a recorder attenuation of 2^5. The results indicate that ligand-exchange chromatography is applicable to the trace determination of L-alanine. The determination of L-glutamic acid in the fermentation medium is also possible.

CONCLUSIONS

The time required for the determination of L-alanine produced by DL-aspartic acid fermentation is about 8 min. The background medium shows no interference with the determination L-alanine and has a minimum effect in analysis with of the fermentation medium dilution. As no derivatization of the amino acids is necessary for detection, sample preparation is simple. Thus, the direct separation of amino acids by using an achiral aqueous mobile phase containing only Zn^{2+} makes the

proposed ligand-exchange chromatographic method fast and convenient. The procedure needs to be improved but it has potential for monitoring amino acid production systems.

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