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Analysis of the enzymatic racemization of D-aspartic acid to L-aspartic acid by the on-line coupling of a solid-phase extraction column and a ligand-exchange high-performance liquid chromatography column

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Abstract

D-Aspartic acid can be enzymatically biotransformed with D-amino acid oxidase and aminotransferase to L-aspartic acid. The reaction was surveyed at three temperatures and a period of 3 days, however, L-aspartic acid can be produced only at the reaction temperature 90°C. However, the separation of D-aspartic acid and L-aspartic acid by ligand-exchange chromatography showed matrix interference. Therefore, the column-switching technique by coupling a solid-phase extraction (SPE) column to the analytical ligand-exchange HPLC column was used to eliminate the matrix effect. The pretreatment of reaction samples with the SPE column was considered as a combination of size-exclusion chromatography and ion-pair chromatography. The ion-pair reagent was 0.005 *M* sodium 1-octanesulfonate aqueous solution adjusted to pH 2.2. Part of the first eluted peak from the SPE column was then switched through the ligand-exchange column and analyzed with a 0.25 m*M* Cu^{2+} aqueous mobile phase of pH 3.6. The quantitative analysis of D- and L-aspartic acids was performed by the standard addition method. Overall, the separation and analysis of D- and L-aspartic acids in the enzymic solution was convenient, fast, and successful with the developed on-line LC–LC column-coupling and column-switching system.

Keywords: Column switching; Enantiomer separation; Solid-phase extraction; Ligand-exchange chromatography; Aspartic acid; Amino acids

1. Introduction

The bioconversion of DL-aspartic acid to L-alanine can be performed by *Pseudomonas dacunhae* [1]. Since this decarboxylation of the β-carboxylic acid group on the aspartic acid is limited only to L-aspartic acid, D-aspartic acid is thus left in the cell culture after the reaction. If the unreacted D-aspartic acid can be separated and undergo a racemization to form L-aspartic acid and then recycled back, the production of L-alanine from DL-aspartic acid would have economic benefits. Although the racemization of amino acid enantiomers can proceed with either concentrated acid or base at high temperature [2,3], we are interested in using enzymes to perform the racemization. This is partly due to the mild conditions and high stereospecificity of the enzyme reaction. Researchers had used racemase [4,5] (e.g., aspartate racemase) at a pH around 7.5 and a temperature of 37°C to perform the racemization of amino acid enantiomers. Recently, a coupled reaction

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with D-amino acid oxidase and aminotransferase to racemize various D-configuration amino acids was reported [6]. Since the particular racemase for the racemization of a particular amino acid enantiomer is usually not easy to obtain commercially, the use of commercially available D-amino acid oxidase and aminotransferase in combination for the biotransformation of D-amino acids to L-amino acids should be practical.

Reports have shown that direct liquid chromatographic resolution of racemic amino acids and their derivatives can be accomplished by a stationary phase based on either crown ether [7,8] or cyclodextrin [9]. However, maintaining a low column temperature and the use of a very acid mobile phase were difficulties met by employing the crown ether-based liquid chromatographic column for the separation of D- and L-amino acid enantiomers. Especially, the low pH value of the mobile phase can cause problems when coupling the column to another liquid chromatographic column. Although many other methods [10–12] also have been used for the analysis of amino acid enantiomers, the simple and direct separation and determination of amino acid enantiomers by an analytical high-performance liquid chromatography (HPLC) ligand-exchange column has been chosen and successfully utilized by us [1].

Recently, the pretreatment of solution sample by solid-phase extraction (SPE) has increased in popularity. Numerous applications [13–21] for the sample analysis by the column-switching technique with on-line coupling of the SPE column to the chromatographic system or capillary electrophoresis system were developed very quickly as well. Therefore, in this paper, an SPE column of LiChrospher RP-4 ADS has been selected for on-line coupling with the ligand-exchange analytical column to pretreat the solution produced by the enzymatic racemization of D-aspartic acid. In this manner, we can get rid of the matrix interference and understand quantitatively and extensively the production of L-aspartic acid from the enzymatic racemization of D-aspartic acid.

2. Experimental

2.1. Materials and chemicals

D-Aspartic acid, L-aspartic acid, DL-aspartic acid, oxaloacetic acid, α-ketoglutarate and aminotransferase clonezyme library including potassium borate buffer at pH 8.0 were purchased from Sigma (MO, USA). D-Amino acid oxidase (E.C. 1.4.3.3), pyridoxal 5′-phosphate, and sodium 1-octanesulfonate were bought from Tokyo Chemical Industry (Tokyo, Japan). Sodium glutamate and anhydrous copper(II) sulfate were obtained from Merck (Darmstadt, Germany). Sodium hydroxide and sulfuric acid were supplied by Shimakyu (Osaka, Japan) and Hayashi (Osaka, Japan), respectively. Water in the laboratory was deionized and distilled before use. Membrane filters of 0.2 μm in pore size was obtained from Advantec MFS (CA, USA). Helium gas of high purity was locally supplied.

2.2. Racemization of D-aspartic acid

About 0.67 g D-aspartic acid, 0.94 g sodium L-glutamate, 1.33 g pyridoxal 5′-phosphate, 21.0 mg D-amino acid oxidase, 0.4 mg aminotransferase clonezyme (Nos. 3 or 4), and 1.25 ml 660 m*M* potassium borate buffer were added to a 250 ml Erlenmyer flask and then made to total solution volume of 50 ml. The flask was stoppered and sealed and a thermometer was inserted into the solution to monitor the solution temperature. The reaction solution was magnetically stirred and heated by a magnetic hot plate. The reaction flask was usually held about 1 cm above the heating surface of the magnetic hot plate in order to avoid direct contact of the heating surface with the bottom of the flask. The solution temperature can be maintained at around 90±2°C in this manner. Other temperatures such as 30 and 60°C were also used for comparison purpose. The reaction period was 3 days. A 5 ml sample before the reaction started and after the end of reaction was used for the analysis. For HPLC analysis the sample was first filtered through a 0.2 μm microporous membrane and then suitably diluted with the mobile phase. The pH of the reaction solutions was also recorded before and after the reaction.

2.3. Analysis of D- and L-aspartic acids by the column-switching technique

The schematic diagram for the column-switching HPLC system is shown in Fig. 1. Two dual piston

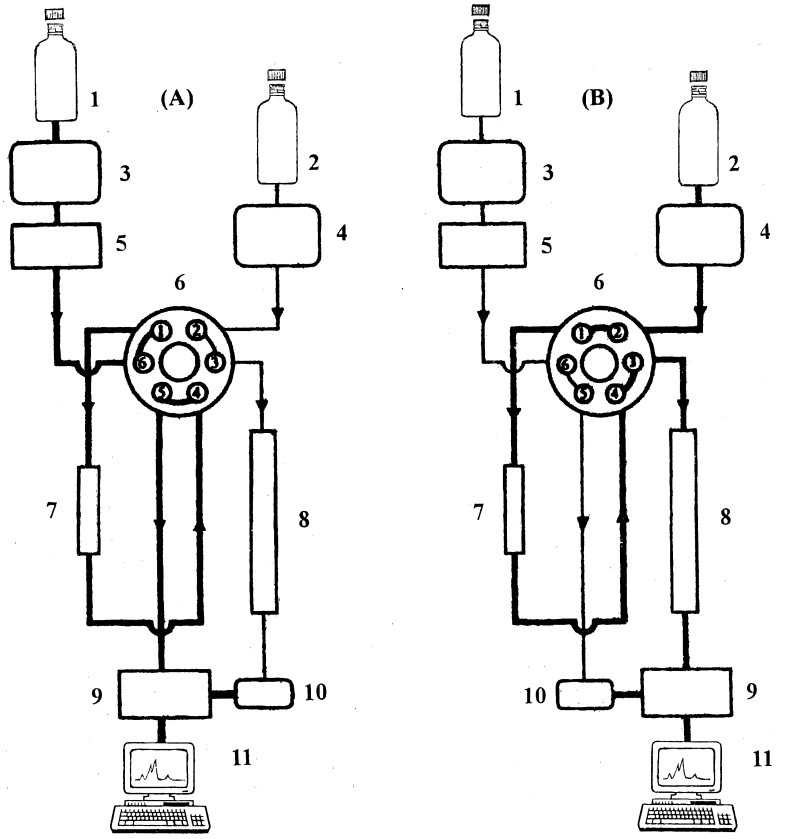

Fig. 1. The schematic diagram for the column-coupling HPLC system. 1=Mobile phase for the SPE column; 2=mobile phase for the analytical column; 3=pump for the SPE column; 4=pump for the analytical column; 5=injection valve; 6=switching valve; 7=LiChroCART 25-4 SPE column; 8=Chiralpak WM analytical column; 9=UV detector; 10=waste container; 11=computer (data station).

solvent-delivery modules (Shimadzu LC-9A, Kyoto, Japan) with a high-sensitivity filter unit were used for pumping two different mobile phases through the SPE column and the analytical ligand-exchange column, respectively. The SPE column was a 25 mm×4 mm I.D. LiChroCART and housed with a special manu-CART (Merck). The sorbent of the SPE column was LiChrospher RP-4 ADS containing a spherical silica gel (25 μm) of two different chemical surfaces. The external surface of the silica gel contains the hydrophilic, electroneutral diol groups and the internal surface of the porous particles has a hydrophobic bonded phase of a C_4 alkyl chain. The external surface protects the sorbent from contamination by proteins and essentially is a size-exclusion chromatography. While the addition of an ion-pair reagent sodium 1-octanesulfonate to the mobile phase may extract and enrich those low-molecular-mass charged compounds to the internal surface. In this way, the SPE column also functions as an ion-pair chromatography. The SPE column temperature was maintained at ambient. The analytical column was a 25 cm×0.46 cm I.D. Chiralpak WM (Daicel, Tokyo, Japan) packed with chiral amino acid modified silica gel (5 μm) that was capable of forming complexes with the Cu^{2+} ion. The injection valve (Rheodyne 7125, CA, USA) with a 20 μl sampling loop was equipped with the pump on the side containing the SPE column. A column oven (Shimadzu CTO-6A) was needed for the analytical column (Chiralpak WM) to maintain a temperature at 45°C. The six-position switching valve (Rheodyne 7000) was used to connect both the two pumps and the two chromatographic columns.

Switching the valve may direct the flow of the two mobile phases through different paths to the columns and to the detector or to the waste. A variable-wavelength UV detector (Shimadzu SPD-6A) at wavelength 254 nm was utilized to detect substances in the solution. The data system consisted of an AMD-K6 3D processor, an ink-jet printer (Epson Stylus color 400, Japan), and the software for processing chromatographic data (Scientific Information Service, Taipei, Taiwan). The running condition of HPLC system was an isocratic flow rate for both mobile phases (0.1 ml min^{-1}, 5 m*M* sodium 1-octanesulfonate aqueous solution at pH 2.2 for the SPE column and 0.5 ml min^{-1}, 0.25 m*M* $CuSO_4$ aqueous solution at pH 3.6 for the analytical column). Helium gas was continuously bubbled through the mobile phases for degassing during the analysis.

2.4. Quantitative method

The standard addition method was applied for the quantitative determination of both D- and L-aspartic acids. Therefore, a series of standard solution containing both D- and L-aspartic acids with concentrations, for example, 3000, 1500, 1000, 750 and 375 ppm were added to the diluted reaction solutions separately. The dilution was made with water. Then each reaction solution including the one without adding any standards was analyzed at least five times by HPLC under the same conditions. A linear calibration curve was obtained by plotting the peak area versus the concentration of the added standards. The slope, the intercept and the linear correlation coefficient of the calibration line were calculated by data processing software. The concentration of the sample solution was thus determined by the ratio of the *y*-axis intercept to the slope of the calibration curve that is in fact the value of the intercept at the *x*-axis [22].

3. Results and discussion

3.1. Racemization of D-aspartic acid with the coupled enzyme system

The reaction solutions were first analyzed by using the ligand-exchange chromatographic column alone. L-Aspartic acid was found in the solution with a racemization temperature 90°C as shown in the two chromatograms of Fig. 2. The pH of the solution containing either aminotransferase No. 3 or aminotransferase No. 4 at the beginning of reaction was 3.9 and 3.7, respectively and it was 4.0 or 3.7, respectively after the reaction. There is no obvious change in the pH for the two solutions tested before and after reaction. The results also elucidated that the coupled enzyme system could perform the racemization of D-aspartic acid in a low-pH medium. Because the search for an optimal pH is not the primary subject in this study, we just temporarily skipped the survey for the optimal pH. Later studies in our laboratory also indicated that L-aspartic acid was produced by heat. From the two chromatograms in Fig. 2 we then discovered that the peak of L-aspartic acid overlaps to some degree both with the peak of D-aspartic acid and the peak on its right. The overlap of peaks in the chromatogram showed the existence of matrix interference. Even though the overlap of peaks is partial, it would cause errors in the quantitative determination of both D- and L-aspartic acids. The resolutions estimated from chromatograms A and B in Fig. 2 for D- and L-aspartic acids were about 0.90 and 0.96, respectively. Since the separation of D- and L-aspartic acids was not ideal, the column-switching technique with on-line coupling of a SPE column and the analytical ligand-exchange column was used to eliminate the matrix effect and to improve the resolution between D-aspartic acid and L-aspartic acid. The use of the on-line column-switching HPLC system in this study has been shown and described in Fig. 1. In both chromatograms of Fig. 2 the key intermediate oxaloacetic acid from the D-aspartic acid by D-amino acid oxidase was mixed with pyridoxal 5′-phosphate as one peak shown in peak 1 and the peak of the key intermediate α-ketoglutarate from L-glutamate was identified as peak 2. The presence of α-ketoglutarate did show the work of the coupled enzymes for the racemization of D-aspartic acid. Amazingly, we found the existence of D- and L-alanine in the reaction solution. However, it is hard to conclude that the coupled enzyme system could also be employed to perform the decarboxylation of the β-carboxylic acid of the aspartic acid.

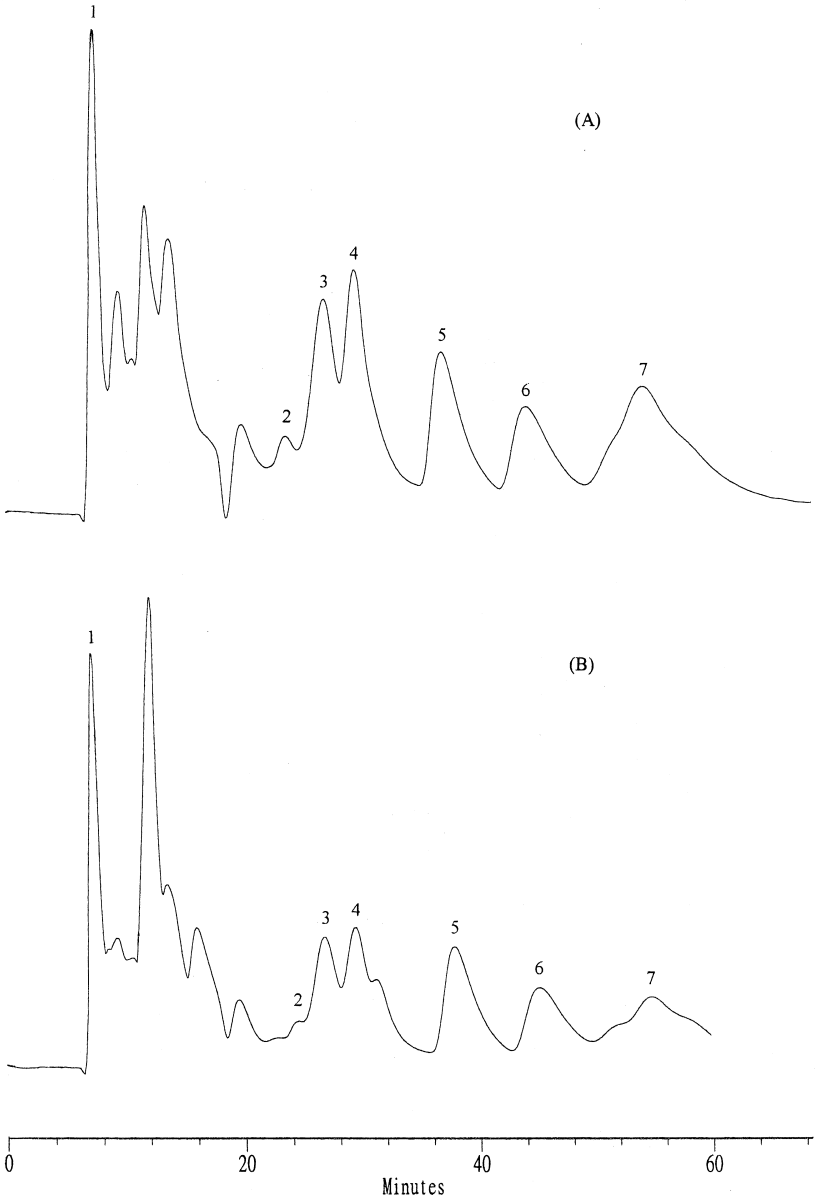

Fig. 2. The analysis of the racemization solution after reaction for 3 days at 90°C by the ligand-exchange chromatographic column. Chromatogram (A) is for solution containing aminotransferase No. 3 and chromatogram (B) is for solution containing aminotransferase No. 4. Peaks: 1=pyridoxal 5′-phosphate and oxaloacetic acid; 2=α-ketoglutarate; 3=D-alanine; 4=L-alanine; 5=D-aspartic acid; 6=L-aspartic acid; 7=sodium L-glutamate.

3.2. Sample pretreatment by the SPE column

The addition of an ion-pair reagent (sodium 1-octanesulfonate) to the aqueous mobile phase for the SPE column was adjusted to a pH 2.2. The choice of this pH is mainly for the protection of the solid-phase extraction column that should be used within a pH range from 2.0 to 7.5. The second consideration for the pH adjustment was the isoelectric point of aspartic acid which is 2.98 [23]. According to this

factor the pH of the solution should be kept as low as possible so that aspartic acid can possess enough positive charge to form an ion pair with the anion of the ion-pair reagent. The concentration of the ion-pair reagent used for this study at the present time was 0.005 *M*. Fig. 3 illustrates the separation of the two reaction solutions containing either aminotransferase No. 3 or aminotransferase No. 4. They were roughly separated into three portions by the SPE column. However, the solution containing aminotransferase No. 4 indicated a better separation for the components. This is probably due to the pH of the solution (3.7), being close to the pH of the mobile phase (2.2). Because the reaction solution was very complex it is not necessary to identify exactly what components exist in the individual peak at this moment. The only thing that interests us is the isolation of the D- and L-aspartic acids from one of the corresponding peaks. Therefore, the next step is the search for the part of the chromatogram including the aspartic acid molecules using the column-switching technique.

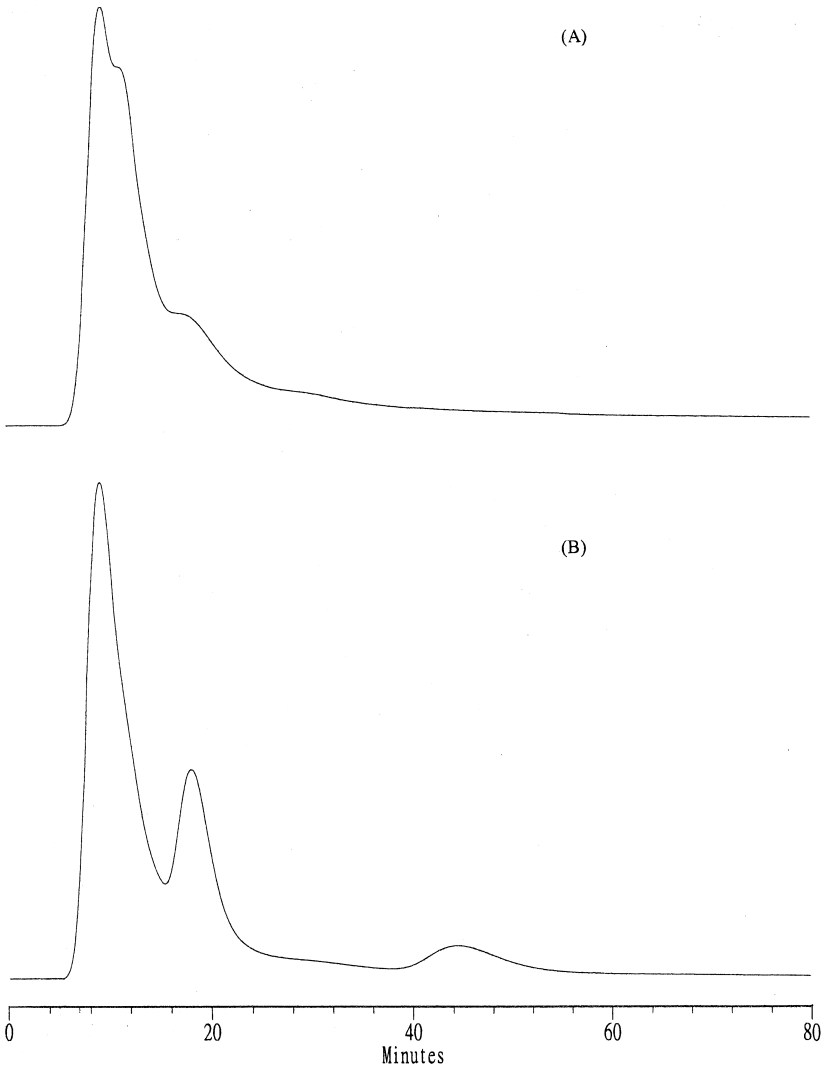

Fig. 3. The pretreatment of the enzymatic racemization solution after reaction for 3 days at 90°C by the SPE column. Chromatogram (A) is the solution containing aminotransferase No. 3 and chromatogram (B) is the solution containing aminotransferase No. 4.

3.3. Analysis by the column-switching technique

In order to select the correct portion of the chromatogram that contains the D- and L-aspartic acids, experiments were performed by dividing the sample into several portions at different time intervals with reference to the roughly separated peaks by the SPE column. Therefore, different portions in chromatogram A of Fig. 3 were selected by the column-switching technique and the resulting chromatograms were shown as chromatograms A–H in Fig. 4. D-Aspartic acid and L-aspartic acid were then found in chromatograms D–F, corresponding to those portions of time interval 5–8, 5–11 and 5–16 min, respectively, in chromatogram A of Fig. 3. In these three chromatograms the partial peak overlap-

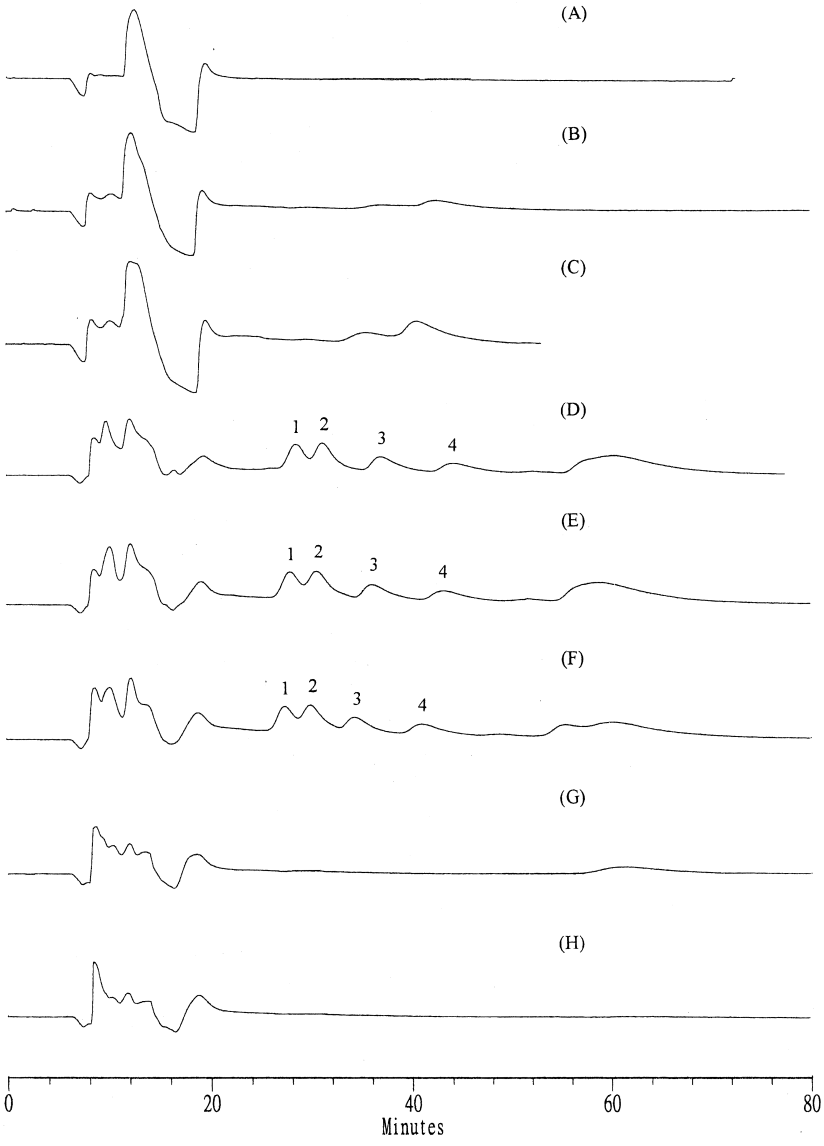

Fig. 4. The analysis of the enzymatic racemization solution containing aminotransferase No. 3 by the ligand-exchange analytical column for portions selected from the SPE column. The time interval of chromatogram (A) 2–3 min; (B) 3–5 min; (C) 4–5 min; (D) 5–8 min; (E) 5–11 min; (F) 5–16 min; (G) 8–11 min; and (H) 11–16 min. Peaks: 1=D-alanine; 2=L-alanine; 3=D-aspartic acid; 4=L-aspartic acid.

ping of the L-aspartic acid that originated from the matrix effect was all eliminated and the resolutions between D- and L-aspartic acids were improved. The values of resolution between D- and L-aspartic acids estimated from chromatograms D–F were 1.45, 1.14, and 1.28, respectively. However, by putting all eight chromatograms in one figure, the *y*-axis (the axis for signal intensity) of the individual chromatogram is pressed in proportion to the number of chromatograms. The corresponding result in the chromatogram is the flattening or broadening of peaks. This result in turn would affect the calculation of resolution between peaks. The calculated resolutions described above thus would be better if we used the original un-pressed individual chromatogram to calculate the peak resolution.

To determine the portion of time interval suitable for the quantitative analysis of D- and L-aspartic acids in chromatogram A of Fig. 3, the significance test was first used to test if a difference exists between the peak areas for both D- and L-aspartic acids at different time intervals. Table 1 lists the peak areas and the standard deviations of D- and L-aspartic acids at different time intervals and they are used for the significance tests. A two-tailed *F*-test was first used to test the equivalence of the two standard deviations and subsequently the Student *t*-value was calculated according to the situation of equivalent or non-equivalent standard deviations of the data. The calculated *t*-value finally was compared with the critical *t*-value at a suitable degree of freedom and certain probability level to determine if a significant difference exists in peak areas between two different time intervals. The results showed that there is no significant difference in peak areas for both D- and L-aspartic acids between the time intervals 5–8 min and 5–11 min or between the time intervals 5–11 min and 5–16 min at the 5% probability level. However, there is a significant difference in peak areas for both D- and L-aspartic acids between the time intervals 5–8 min and 5–16 min. These results mean that the small difference in the duration time from the column switching has no significant effect on the peak areas for both D- and L-aspartic acids. Looking at chromatogram F in Fig. 4, the peak of D-aspartic acid is convoluted a little bit with the peak in front of it. In addition, we can find that during the time interval 5–16 min in chromatogram A of Fig. 3 there actually involves two peaks. We thus suspect that the substances in the second peak could affect the quality of separation and thus the quantitative analysis. The peak areas for both D- and L-aspartic acids in the time interval 5–11 min in Table 1 possess the largest relative standard deviations (RSDs) and they possess the smallest RSDs in the time interval 5–8 min. Therefore, substances in the second peak of chromatogram A in Fig. 3 did affect the precision of the analysis. Because we cannot detect D- and L-aspartic acids in the portion of time interval 8–11 min as shown in chromatogram G of Fig. 4, the loss of D- and L-aspartic acids by switching the valve at 8 min can be neglected. Thus, we decided to use the portion of the sample switched at the time interval 5–8 min for the subsequent quantitative analysis of D- and L-aspartic acids.

Similarly, portions in chromatogram B of Fig. 3 were selected and passed through the analytical ligand-exchange column by the column-switching technique. The resulting chromatograms A–I with different time intervals are shown in Fig. 5. D- and L-aspartic acids were found in chromatograms B–G that correspond to the time intervals 3–4, 3–5, 4–5, 4–8, 4–15.5 and 4–20 min, respectively. The resolutions between D- and L-aspartic acids estimated

Table 1
Peak areas and standard deviations of D-aspartic acid and L-aspartic acid at different time intervals for the reaction solution containing aminotransferase No. 3

	5–8 min		5–11 min		5–16 min	
	D-Aspartic acid	L-Aspartic acid	D-Aspartic acid	L-Aspartic acid	D-Aspartic acid	L-Aspartic acid
Average[a] peak area	721 682.0	586 677.4	736 342.0	600 293.8	750 509.0	610 243.0
SD peak area	6609.2	3556.2	54 359.8	23 166.1	22 636.8	15 333.9
RSD (%)	0.92	0.61	7.38	3.86	3.02	2.51

[a] The average value was calculated from five or six data.

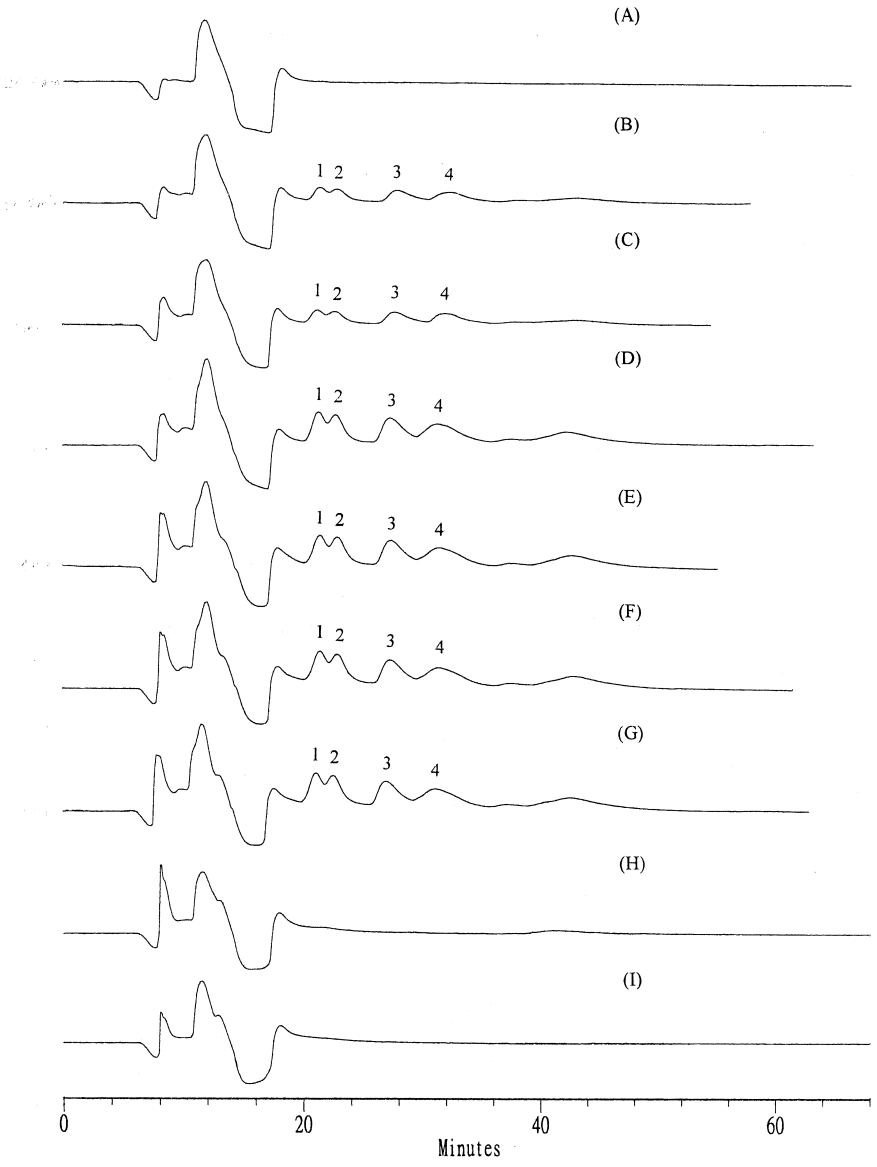

Fig. 5. The analysis of the enzymatic racemization solution containing aminotransferase No. 4 by the ligand-exchange analytical column for portions selected from the SPE column. The time interval of chromatogram (A) 2–3 min; (B) 3–4 min; (C) 3–5 min; (D) 4–5 min; (E) 4–8 min; (F) 4–15.5 min; (G) 4–20 min; (H) 8–15.5 min and (I) 15.5–30 min. Peaks: 1=D-alanine; 2=L-alanine; 3=D-aspartic acid; 4=L-aspartic acid.

from the corresponding chromatograms were 1.02, 0.93, 0.82, 0.82, 0.83 and 0.88, respectively. Although the resolutions did not all improve, the matrix effect was reduced a lot. We also have to mention that the *y*-axis is pressed to cause peak flattening and the flattening of peak may decrease the resolution a little bit. However, the main reason that the resolution has not been improved was due to the deterioration of the ligand-exchange analytical column by the low-pH mobile phase. Peak areas for both D- and L-aspartic acids in the two time intervals 3–4 min and 3–5 min were all small thus these two

Table 2
Peak areas and standard deviations of D-aspartic acid and L-aspartic acid at different time intervals for the reaction solution containing aminotransferase No. 4

	4–5 min		4–8 min		4–15.5 min		4–20 min	
	D-Aspartic acid	L-Aspartic acid	D-Aspartic acid	L-Aspartic acid	D-Aspartic acid	L-Aspartic acid	D-Aspartic acid	L-Aspartic acid
Average[a] peak area	1 059 183.0	1 212 259.2	1 034 434.8	1 161 047.6	1 046 833.0	1 133 495.2	936 214.4	1 039 240.0
SD peak area	8389.9	13 444.9	13 071.6	39 072.0	24 717.8	16 760.1	84 354.2	91 202.9
RSD (%)	0.79	1.11	1.26	3.37	2.36	1.48	9.01	8.78

[a] The average value was calculated from five or six data.

portions of solution are not considered suitable for the quantitative analysis. Therefore, differences between the peak areas for both D- and L-aspartic acids in the four chromatograms with switching time intervals of 4–5, 4–8, 4–15.5 and 4–20 min were tested by the significance test at a 5% probability level as described previously. The peak areas and the standard deviations of D- and L-aspartic acids for the above four portions of reaction solution are shown in Table 2. There is no significant difference in peak areas for D-aspartic acid between the time intervals 4–5 and 4–15.5, 4–8 and 4–15.5, and 4–8 and 4–20 min. The peak areas for L-aspartic acid between the time intervals 4–8 and 4–15.5 min and between the time intervals 4–15.5 and 4–20 min do not show significant difference and all other intervals show significant difference. These results indicated that the time intervals of 4–8, 4–15.5 and 4–20 min are closely related to each other but not to the time interval 4–5 min. Also, in chromatogram B of Fig. 3 the time intervals of 4–15.5 min and 4–20 min include the second peak coming from the SPE column. Since we do not like to risk possible interference from the second peak we would rather choose the portion with the time interval including only one peak coming from the SPE column for the subsequent quantitative analysis. Therefore, we decided to use the time interval 4–5 min which possesses the smallest RSDs for both D- and L-aspartic acids for the quantitative analysis.

3.4. Quantitative results

The results above proved that the separation for D- and L-aspartic acids in the enzymatic racemization solution by the column-switching technique with the on-line coupling of a SPE column and an analytical ligand-exchange column was successful. The quantitative analysis for D- and L-aspartic acids in the reaction solution containing aminotransferase No. 3 was performed by the standard addition method by adding 200, 100, 50, 25 and 12.5 ppm standard D- and L-aspartic acids to different aliquots of 50-fold diluted solution. The concentrations for D- and L-aspartic acids were then calculated by the calibration curve to give 3102 ± 29 ppm and 2279 ± 88 ppm, respectively. Similarly, the quantitative results for D- and L-aspartic acids in the reaction solutions containing aminotransferase No. 4 were 2741 ± 68 ppm and 2476 ± 46 ppm, respectively. The original amount of D-aspartic acid used for the reaction was about 13 000 ppm and the total amount of D- and L-aspartic acids after the reaction was around 5300 ppm. The difference between the original amount and after the reaction was that part of the D-aspartic acid was consumed by the side reactions to side products such as D- and L-alanines. Since none of the RSDs of the analytical results exceeded 4.0% the analysis was quite precise.

4. Conclusion

The racemization of D-aspartic acid to L-aspartic acid performed in this study was enzymatic and with two coupled enzymes (i.e., D-amino acid oxidase and aminotransferase clonezyme). However, later studies showed that the racemization can be proceeded at high temperature by heat alone. The use of D-amino acid oxidase without coupling to the aminotransferase may also help the production of L-aspartic acid. However, the yield of the L-aspartic acid was greatly increased by the use of coupled enzyme system. The matrix effect of the reaction solution for the analysis of D- and L-aspartic acids can be eliminated by the column-switching technique with on-line coupling of a SPE column to the HPLC system. The analysis of D- and L-aspartic acids by the ligand-exchange column was thus performed efficiently with the selection of suitable portion of the reaction solution through the SPE column. In general, the larger size enzyme molecules are excluded first from the SPE column then sequentially smaller molecules are retained in different degrees by the SPE column according to the conditions of ion-pair chromatography. After the reaction solution has been separated into different parts by the SPE column, the part containing D- and L-aspartic acids can be selected and switched to the ligand-exchange analytical column successfully. The matrix interference between D- and L-aspartic acids was thus eliminated in the ligand-exchange column and the quantitative analysis was performed precisely. Studies on the separation conditions of the SPE column such as the concentration of the ion-pair reagent, the pH of the

mobile phase, and the flow rate should be surveyed further in order to get an optimum value. Overall, the present development of the on-line LC–LC column-coupling with the column-switching system on the separation and analysis of D- and L-aspartic acids in the enzymic solution was convenient, fast, and successful.

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