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Aspartase-Catalyzed Preparative Scale Synthesis of ^{15}N-Aspartic Acid

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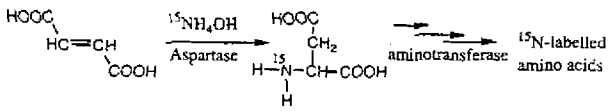
A preparative scale synthesis of ^{15}N-aspartic acid from fumarate and $^{15}NH_4OH$ catalyzed by immobilized aspartase has been developed. Immobilization of aspartase in membrane enclosed enzyme immobilization (MEEI) facilitates the separation of the enzyme from product and makes the enzyme stable and durable for multiple usage. A simple isolation procedure in total yield of 62% using perfusion ion exchange chromatography renders the procedure more practical.

INTRODUCTION

Aspartase (EC 4.3.1.1) catalyzes the reversible conversion of fumarate and ammonia to L-aspartate, which is a component of the sweetener Aspartam. Increased interest has been focused on aspartase, with researchers attempting to engineer for increasing enzymatic stability,[1] to study the physiological roles of the enzyme,[2,3] to understand the acid-base chemical mechanism,[4,5] and to clone the gene for overexpress.[6-8] A preparative scale procedure using the enzyme will be a practical way to synthesize aspartic acid. ^{15}N-Labeled amino acid is very useful in studying the complexes structure of peptides associated with other biologically active compounds.[9] The ^{15}N-Labeled amino acids can be obtained from ^{15}N-Labeled aspartic acid catalyzed by aspartate aminotransferase.[10-13] As part of a program we like to prepare ^{15}N-Labeled amino acid derivatives for determining the structures of peptide or protein when associated with other organic compounds. ^{15}N-Labeled aspartic acid is needed to synthesize other ^{15}N-Labeled amino acid derivatives. We describe here a preparative scale synthesis of ^{15}N-aspartic acid catalyzed by aspartase. No other practical procedure using aspartase-catalyzed synthesis of L-aspartic acid has yet been reported. This procedure is also applicable to the synthesis of aspartic acid from fumarate and ammonia (Scheme I).

Scheme I



EXPERIMENTAL PARTS

Materials and Methods

Aspartase was purchased from Sigma Biochemicals Co., (USA) with a specific activity of 1.7 unit.mg^{-1}. It was used without further purification. The amino acids were purchased from Sigma, USA. ^{15}N-Ammonium hydroxide (98% isotope purity) was purchased from Cambridge Isotope Lab. (USA). Chiral HPLC column MA(+) was purchased from Diacel, (USA). DEAE Hyper DF column was purchased from BioSepra Inc.(France). Common organic solvents, HPLC grade and reagent grade, were obtained from a local supplier, the ALPS Chem. Co., (Taiwan).

Enzymatic Activity of Aspartase

Enzymatic activity of aspartase at 25 °C was determined by spectrophotometric measurement of the increased absorbance at 240 nm (ε_{240} = 2.53 mM^{-1}cm^{-1}) due to the formation of fumarate. All assays were run on U-3300 spectrophotometer, Hitachi, in 3-mL total volume in a 1-cm path length cuvette. The assay mixture contained 50 mM Tris-buffer pH 8.5; 50 mM L-aspartate; 2 mM $MgSO_4 \cdot 7H_2O$; 0.1 mM EDTA. Unless stated otherwise, all reactions were initiated by the addition of aspartase.

Immobilization of Aspartase

Aspartase was immobilized in the dialysis bag (cut off Mw 12,000) by dissolving the enzyme in phosphate buffer (5 mM, pH 7.5) and added into the bag according to the procedure of Bendnarski *et al.*[14]

Stability of Aspartase

The stability of free and immobilized aspartase in reaction solution was determined by measuring the initial rates of incubated enzyme as the remaining activity of the aspartase at different time. In a typical reaction, 0.1 mg of

aspartase in reaction solution (3 mL) was stirred at 25 °C to maintain homogeneity. Periodically, 50 μL aliquots were taken and added to a curette containing assay mixture. The initial reaction rates were determined from time-dependent plots of the increasing absorbance at 240 nm. The 100% activity point was taken to be 10 seconds after the addition of aspartase to the incubation solution.

Preparative Scale Synthesis and Isolation of ^{15}N-L-Aspartic Acid

To a mixture of fumaric acid (17.4 g, 150 mM), ^{15}N-ammonium hydroxide (30 mL, 100 mM), $MgSO_4$ (0.60 g, 5 mM), and NaN_3 (0.05%) dissolved in 1.0 L of the reaction solution (pH = 7.5) was added the immobilized aspartase. Reaction was monitored by HPLC. After the concentration of ^{15}N-L-aspartic acid was no longer increased (~9 days), the ^{15}N-L-aspartic acid was isolated and purified by preparative HPLC; Column: DEAE Hyper DF (1.0 × 25 cm); Eluent: 0.1 M NaH_2PO_4/0.1 M NaCl, pH = 4.25, Flow rate: 4 mL/min, Detection: UV 210 nm. ^{15}N-L-aspartic acid (8.32 g) was isolated with 62% yields. ^{1}H NMR (D_2O, 400 MHz): 2.65 (m, 2H), 3.78 (m, 1H). ^{13}C NMR (D_2O, 100 MHz): 33.40 (C-3), 48.89 (C-2), 171.71, 174.38. ^{15}N NMR (D_2O, 40.5 MHz): -345.11 ($^{15}NH_2$). ^{15}N-L-aspartic acid has the same isotope purity (98%) as that of the starting material $^{15}NH_4OH$ by comparing the integration of their ^{1}H NMR spectra in which the chemical shift of $^{14}NH_2$ is 8.34 and $^{15}NH_2$ is shown doublet peak at 8.27 and 8.46.

RESULTS AND DISCUSSION

Fig. 1 shows the reactivity of aspartase at various pH values. The enzyme is very active at pH = 7.0 and 7.5. At pH = 9.0 or 5.0 the enzymatic activity is only 85% of that of the optimal pH. The stability of aspartase was studied by incubation of the enzyme in the reaction solution at pH levels from 5.0 to 9.0 for a certain period and measurement of the remaining activity of the incubated enzyme. The enzyme incubated at pH = 7.0 and 7.5 was more stable than that incubated at other pH levels. The aspartase maintained 70% of its original activity, after being incubated for one week at pH = 7.0 and 7.5. Fig. 2 shows the remaining activity of the aspartase after being incubated at various temperatures at pH = 7.5. The aspartase maintained 85% of its original activity up to 50 hours, and the stability of the enzyme decreased as the temperature increased. At 40 °C the enzyme had a half life of 4 hours, and at 25 °C of more than 120 hours.

To maintain stability of the enzyme and facilitate the separation of the product from aspartase, the stability and durability of immobilized aspartase via the membrane enclosed enzyme immobilization (MEEI) method was investigated. Table 1 shows the durability and the remaining activity of MEEI aspartase after use and re-use. The immobilized aspartase maintained 40% of its original activity for one month after being used three times. We reasoned that aspartase is a tetramer which easily loses its enzymatic activity in the dissociate state. The MEEI kept the aspartase with high concentration in the dialysis bag and prevented the dissociation of the enzyme. We also observed that the enzyme lost activity quickly when incubated in dilute solution.

Preparative scale synthesis of ^{15}N-L-aspartic acid was carried out in 1.0 L reaction solution containing fumaric acid (150 mM), ^{15}N-ammonium hydroxide (100 mM), and MEEI aspartase (50 mg in 20 mL phosphate buffer, pH = 7.5). Fig. 3 shows the time course for the production of ^{15}N-L-aspartic acid in the reaction solution. After incubation for

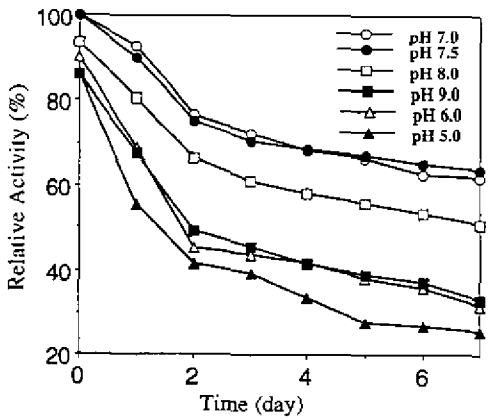


Fig. 1. Residue activity of aspartase after incubation at various pH levels.

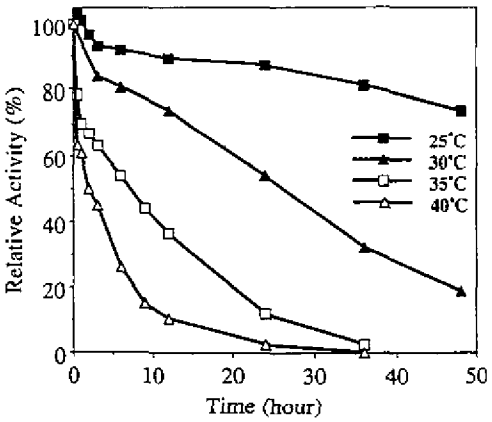


Fig. 2. Residue activity of aspartase after incubation at various temperatures.

Table 1. Stability of Aspartase after Re-used

Time	remaining activity (%)
Used 1 time (9 days)	69.80
Used 3 times (1 month)	40.93

9 days, the concentration of the ^{15}N-L-aspartic acid increased to about 76 mM and no longer increased. The reaction was stopped and the product was isolated by perfusion chromatography using DEAE ion exchange column.

Aspartic acid and fumaric acid are dicarboxylic acids and difficult to separate by the traditional method. Using perfusion chromatography, mixtures of aspartic acid, fumaric acid, and malic acid can be separated. Fig. 4 shows the chromatography of a mixture of aspartic acid **1**, fumaric acid **3** and malic acid **2** in a ratio of 6:1:2 (w/w). Malic acid

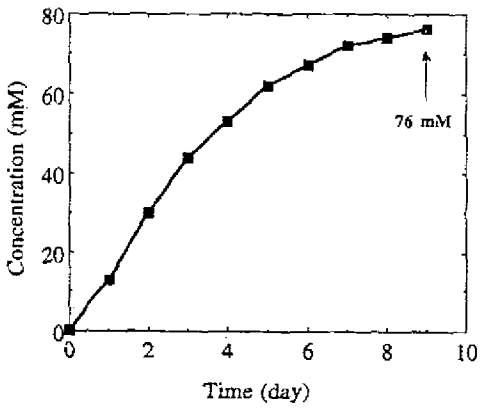


Fig. 3. Time course for aspartase-catalyzed reaction.

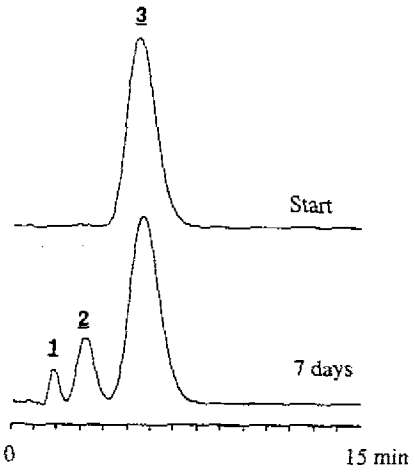


Fig. 4. Perfusion HPLC of Aspartic acid **1**, malic acid **2**, and Fumaric acid **3**. Column: DEAE Hyper D, Eluent: 0.1 M NaH_2PO_4/0.1 M NaCl pH 4.25, Flow rate: 1 mL/min, Detection: UV 210 nm.

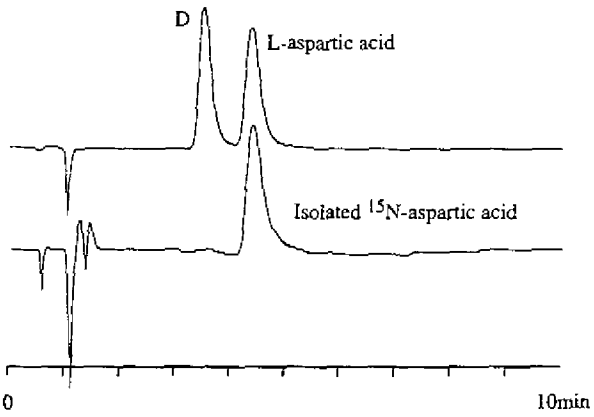


Fig. 5. Chiral separation of D&L-Aspartic acid and isolated product, Column: MA(+) (0.46 × 5 cm), Eluent: 10 mM $CuSO_4$, Flow rate: 1 mL/min, Detection: UV 254 nm.

sometimes appeared in the reaction solution due to the hydration of fumaric acid. From the 76% conversion of the product, ^{15}N-L-aspartic acid was isolated with 62% yield by a preparative perfusion column (1.0 × 25 cm). Optical purity of the synthesized ^{15}N-L-aspartic acid was measured using a Chiral MA (+) column. Fig. 5 shows the results of the chromatography. The isolated product contained only optically pure ^{15}N-L-aspartic acid.

Isotope labelled compounds are useful for the investigation of enzyme-catalyzed stereospecific reactions,[15] for metabolism pathway studies,[16] and for tracing the biosynthesis process.[17,18] In conclusion, this is the first report of a preparative scale synthesis of optically active L-aspartic acid catalyzed by aspartase. The simple procedure and the intensive study in many laboratories to obtain enough quantity of the enzyme by molecular biology technology will lead to large scale production of aspartic acid by the enzymatic method instead of by fermentation. In conclusion, the current procedure is not only applicable to synthesis of ^{15}N-L-aspartic acid but also to large scale synthesis.

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Key Words

^{15}N-Aspartic acid; Aspartase; Immobilization.

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