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FACILE SYNTHESIS OF L-MALIC ACID BY A CONSECUTIVE ENZYMATIC REACTION.

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Procedures for a consecutive reaction catalyzed by fumarase and followed by aspartase in ammonium fumarate buffer to facilitate the separation of product have been developed. Fumarate was converted to L-malate in 83%, and the rest of the starting material was converted to aspartate in 16.7% to make a total conversion of 99.7%. After the solvent was evaporated, the malic acid was dissolved in acetone, and the aspartic acid was insoluble. A simple filtration could separate both products.

Introduction

Optically pure malic acid is one of the versatile chiral synthons for synthesis of various chiral compounds [Norman & Morris, 1992.; Buser et al., 1991; Ramos & Bellus, 1991] Many procedures have been developed for preparinmg L- or D-malic acid [Yamamoto et al., 1976; Ushio et al., 1992; Miyama & Nakayama, 1993]. Racemic malic acid can be simply prepared by the hydration of fumaric acid in aqueous solution, and optically active L-malic acid can be prepared by an enzymatic procedure using immobilized microbial cells [Yamamoto et al., 1976]. One drawback for the enzymatic procedure has been the incomplete conversion of the fumarate to malate. The equilibrium constant for converting fumarate to malate was about 0.23 [Bock & Alberty, 1953; Massey, 1953; Brant et al., 1963]. Thus, only 80% of the fumarate could be converted to the malate and the remaining 20% of the starting material in the reaction mixture made the product hard to separate due to the similar chemical and physical properties of both compounds. We describe here a simple consecutive enzyme reaction by which the conversion of fumarate was nearly complete (99.7%), yielding malate (83%) and aspartate (16.7%). After acidification and evaporation of the solvent, the malic acid could dissolve in acetone, and the aspartic acid was insoluble; thus, malic acid could be simply separated by a filtration with high yield and enantiomeric excess.

Experimental

Aspartase (from Hafnia alvei, ATCC 9750, 300-500 units per mg.), and Fumarase (from porcine heart, E.C. 4.2.1.2., 300-500 units per mg.) was purchased from the Sigma Chem Co., U.S.A. It was used without further purification. Fresh fumarase (from porcine heart, 250 unit/mL) was isolated in accordance with the procedure of Beeckmans & Kanarek, (1977). Optical rotation was measured on a Universal Polarimeter (Schmidt & Haensch, Germany). The hplc system consisted of two Waters model 6000 pumps, a Waters Model 450 uv detector, and an M-660 solvent programmer.

A Suntex P.C. 303 Auto-pH Controller was used (the Suntex Instruments Co., Taiwan). Tlc was performed on silica gel G pre-coated plates (E. Merck., Germany). Methanol, ethyl acetate, methylene chloride, acetonitrile, dioxane, ether, and acetone (hplc grade and reagent grade) were obtained from a local supplier, the ALPS Chem. Co. (Taiwan).

Enzyme Assay.

Enzymatic activities of fumarase were measured spectrophotometrically by measuring the absorbency increase at 240 nm due to the conversion of malate to fumarate. The assays were performed at pH 7.6 at 25°C in phosphate buffer (0.05 M). One unit converted 1.0 μmole of L-malate to fumarate per min at pH 7.6 at 25°C [$\Delta\varepsilon_{240nm} = 2400\ M^{-1}CM^{-1}$].

Stability of fumarate and aspartate in ammonium fumarate buffer.

The stability of fumarate and aspartase in ammonium fumarate was determined by spectrophotometric measurement of the absorbancy rise at 240 nm due to generation of fumarate from the substrate. In a typical reaction, an enzyme solution (fumarase, 0.05 mM or aspartase 0.05 mM) in ammonium fumarate (0.05 M, pH 7.6) was stirred at 25°C to maintain homogeneity. Periodically, 100 μl aliquots were taken and added to a cuvette containing malate (0.10 mM for fumarase) or aspartate (0.15 mM for aspartase) in 0.2M phosphate buffer (pH 7.6 at 25°C). The initial reaction rates were determined from time-dependent plots of the increasing absorbancy at 240 nm. The 100% activity point was taken to be 10 seconds after the addition of the enzyme to the ammonium fumarate solution.

Preparative synthesis of malic acid.

Fumaric acid (11.6 g., 100 mmole) was dissolved in water (50 mL) by the addition of ammonium hydroxide (25% solution) on a stirrer. The solution was adjusted to pH=7.6 and diluted to reach a final volume of 100 mL. To the resulting solution was added fumarase (2.0 mg, 600U) to start the reaction. The mixture was stirred at 25°C for about 12 hours, and the resulting mixture was then filtered using ultrafiltration (mw 8000 cut-off membrane) to remove fumarase. To the filtrate was added aspartase (1.5mg, 450 U), and the reaction was continued for a further 12 hours. The aspartase was separated from the reaction solution by ultrafiltration, and the filtrate was acidified to pH 1.0 with 6 N HCL. The resulting solution was evaporated by a lyophilizer to yield a white powder, which was suspended in acetone (300 mL). The soluble part of the malic acid in acetone was separated by filtration, and the organic solvent was evaporated under reduced pressure. Malic acid was obtained as a white amorphous solid (9.63g, 83%): mp: 97.5-99°C; $[\alpha]^{25}_{D} = -3.36$, (c 2, MeOH);

Results and Discussion

The stability of the enzymes in aqueous solution was investigated. Figure 1 shows the stability of aspartase (Fig 1-a), fumarase (Fig 1-b,) and fresh isolated fumarase (Fig. 1-c). The aspartase could maintain 60% of the original activity within 24 hours, and the commercial fumarase could maintain 40% of the original activity for more than three days. The fresh isolated fumarase could maintain 50% of its original activity for more than three weeks.

Scheme I shows the consecutive enzyme reaction. In order to avoid salt or ions that might contaminate the products during the isolation, the reaction was carried out in ammonium fumarate solution instead of the traditional buffer solution. The fumaric acid was dissolved in water, and the aqueous solution was adjusted to pH 7.5 with ammonium hydroxide (25%). The course of the reaction was monitored using an hplc. After the reaction finished, the enzyme was removed from the reaction solution by ultra-filtration.

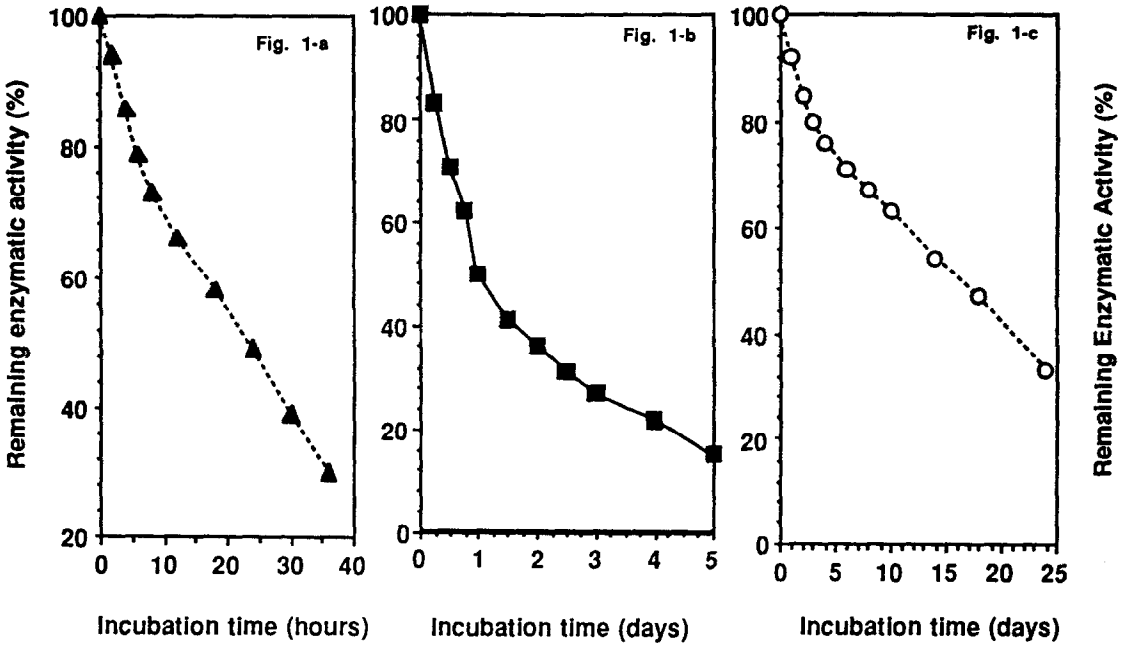

Figure 1. Stability of aspartase (1-a), fumarase (1-b), and fresh isolated fumarase (1-c).

Scheme-I

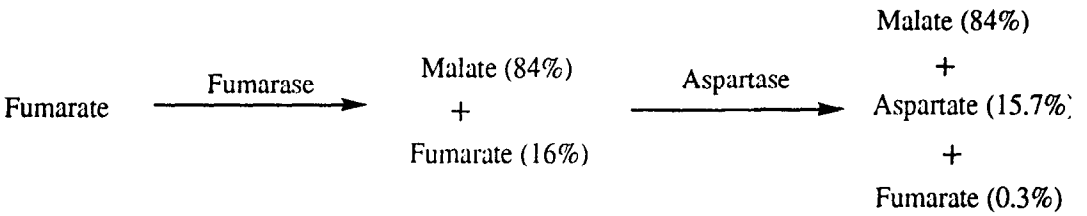

Figure 2 shows the time course for the consecutive enzyme reaction in 1M fumarate solution. After reaction for 12 hours, the concentration of fumarate was decreased to 14.7% and did not decrease any more. The fumarase was separated via ultra-filtration, and to the filtrate was added aspartase to convert the rest of the fumarate to aspartate. The reaction was continued for a further 12 hours, and a final composition of fumarate, 0.058%, aspartate, 14.65% and malate, 83.7% was reached as measured by hplc. The aspartase was removed by ultra-filtration, and the filtrate was acidified to pH 1.0 and then lyophilized to obtain a crude product, which was redissolved in acetone to dissolve the malic acid and filtrated to remove insoluble aspartic acid. The filtrate was evaporated to yield L-malic acid.

There are some special features of this process: i). using ammonium fumarate as buffer eliminates contamination of the buffer during isolation; ii) the ammonium ion is also one of the substrates of aspartase at the second step; iii) the presence of malate in the reaction solution will not

affect the conversion of fumarate to aspartate in the second reaction.

Recent progress in industrial separation of carboxylic and amino acids by liquid membranes [Eyal & Bressler 1993] led us to consider the possibility of using a consecutive enzymatic reaction. In practice, using the solubility difference of both products may be more convenient for separating the products. In conclusion, this procedure demonstrates that a consecutive enzymatic reaction can facilitate the synthesis of malic acid. The stability of aspartase and fumarase enables complete conversion in the reaction.

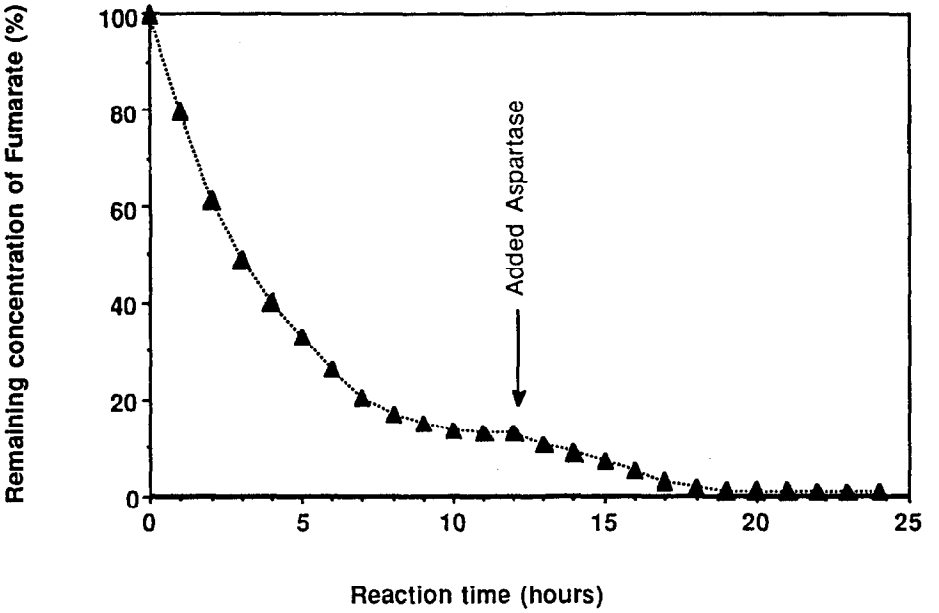

Figure 2. Time course for the consecutive enzymatic reaction catalyzed by fumarase followed by aspartase.

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