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CONFIRMING CHIRAL HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS WITH STEREOSPECIFIC ENZYMES

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SUMMARY

Identification of optical isomers of amino acids, separated by chiral high-performance liquid chromatography, has proved to be difficult. Despite highly selective separation techniques, identification of peaks based on retention times alone is usually uncertain, particularly in assays of complex biological samples in which interfering compounds are eluted with the peak of interest. We describe here an approach for increasing the certainty of identification of peaks of D- and L-amino acids by the use of stereospecific amino acid oxidase and racemase enzymes. A portion of the sample is first incubated with the enzymes. The amino acids in the treated and untreated portions are then chromatographed and the chromatograms of the samples with and without enzyme incubation are compared. The differences are used to help identify the amino acids.

INTRODUCTION

We have described several effective methods for the high-performance liquid chromatographic (HPLC) separation of optical isomers of amino acids after derivatization with dansyl chloride[1] or with *o*-phthalaldehyde in the presence of N-acetyl-L-cysteine[2]. In applying these techniques for assaying D- and L-pipecolic acids in the urine of patients with an inborn error of amino acid metabolism[3], we noted that these compounds, which were present in low concentrations, were eluted at approximately the same time as other minor components of the mixture and could not be reliably be distinguished from them. Retention time was not specific enough; it helped only to locate the amino acid. Before stereoselective chromatographic procedures were developed, D-, L-specific amino acid oxidases were used to distinguish optical isomers[4,5]. The amino acid oxidases, which react with amino acids either in the D or L series, are only semi-specific. Earlier, the enzymatic procedure was often performed in conjunction with ion-exchange chromatography if more than one amino acid is expected in the mixture. Recently, an entirely different approach for improving the confidence of D and L optical isomer identification involving an optical activity detector was introduced by Reitsma and Young[6,7]. In the work presented here, the enzyme-assisted assay was performed in conjunction with a reversed-phase HPLC procedure that effectively resolves racemic mixtures of most amino acids but is unable to separate all possible amino acid metabolites in biological mixtures.

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EXPERIMENTAL

Reagents

Methanol and acetonitrile, "distilled-in-glass" quality, were bought from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Amino acids and D-amino acid oxidase were purchased from Sigma (St. Louis, MO, U.S.A.). The mobile phases generally contained various concentrations of acetonitrile in buffer (pH 7.0), containing 5.0 m*M* of L-proline, 2.5 m*M* of copper sulfate and 2.0 g/l of ammonium acetate.

Instrumentation

The HPLC system consisted of two Altex (Berkeley, CA, U.S.A.) 110A pumps, an Altex 420 gradient microprocessor and a Rheodyne (Cotati, CA, U.S.A.) 7105 injection valve. The analytical column, 15 × 0.42 cm I.D., was packed with Nucleosil 5 C_{18} (Duren, F.R.G.) by the downward slurry technique. The dansyl amino acids were detected with a Fluoro-tec filter fluorometer (American Research Products, Kensington, MD, U.S.A.). The amplified detector signals were read out on a Model 4416 data system (Nelson Analytical, Cupertino, CA, U.S.A.).

Reaction with D-amino acid oxidase

To assay the activity of the enzyme, a stock solution containing 2 mg D,L-alanine in 1 ml of 0.02 *M* potassium pyrophosphate (pH 8.3) was prepared. To 1 ml of this solution was added 200 μl of a solution containing 20 mg of D-amino acid oxidase and 5 mg of catalase in 1 ml of same buffer. The mixture was incubated at 37°C. The reaction of D-alanine with the enzyme was followed as a function of time by withdrawing 100-μl aliquots of the substrate every 15 min, synthesizing the dansyl derivative and assaying by HPLC. Dansylation was effected by adding 100 μl of the substrate to 400 μl of lithium carbonate followed by 200 μl of dansyl chloride (1.0 mg/ml in acetonitrile). At 100°C, dansylation was complete in 20 min. The same procedure was used to treat an aliquot of the complex mixture to be assayed.

Reaction of amino acid with racemase

A 10-ml aliquot of a *S. faecalis* culture was centrifuged at 3000 *g* for 15 min, and the cells were harvested, after three washings with 2-ml portions of isotonic saline. To the cells were added 2 ml of phosphate buffer (pH 8.1), 200 μl of pyridoxal phosphate (1 mg/ml) and 1 ml of D,L-alanine (2 mg/ml). The cells were then ruptured by ultrasonication in the presence of glass beads and the mixture was incubated at 37°C. To monitor the course of the reaction, 100-μl portions were removed every 30 min, dansyl derivatives of the amino acids were prepared, and aliquots were assayed by HPLC.

RESULTS

When a mixture of D,L-alanine was incubated with D-amino acid oxidase, the peak corresponding to the D-isomer gradually decreased in size as the D-alanine was oxidatively deaminated (Figs. 1 and 2). This was taken as confirmation of the separation of the D- and L-isomers and of the identification of the D-isomer by its

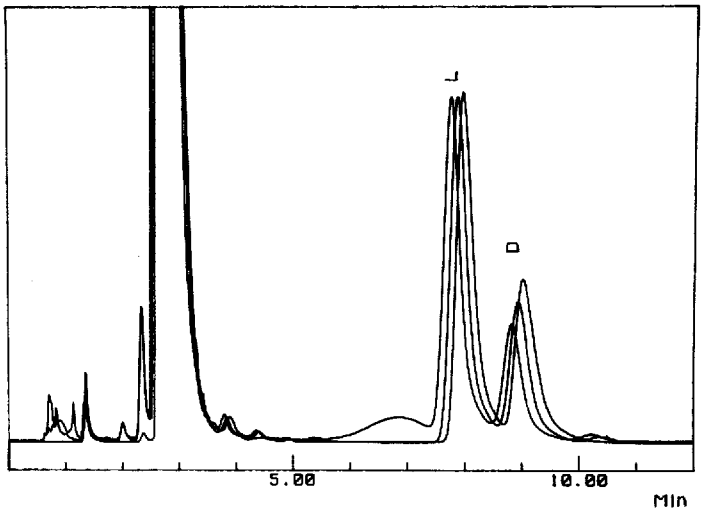

Fig. 1. Chromatogram of D,L-alanine. The size of the D-alanine peak decreased gradually as D-alanine was oxidatively deaminated at 0, 1 and 2 h. Mobile phase: 15% acetonitrile in a buffer (pH 7.0) containing 5 m*M* L-proline, 2.5 m*M* $CuSO_4 \cdot 5H_2O$ and 2.0 g ammonium acetate. Flow-rate, 2.0 ml/min.

retention time. With the enzyme used, there was no detectable reaction with the L-isomer. Incubation of a synthetic mixture, containing many D- and L-amino acids, with D-amino oxidase, followed by chiral HPLC, similarly showed marked diminution in size of the D-isomers (Fig. 3).

When a racemic mixture of alanine was incubated with the cell extract of *S. faecalis*, the L-isomer was racemized to the D-isomer until a constant proportion was

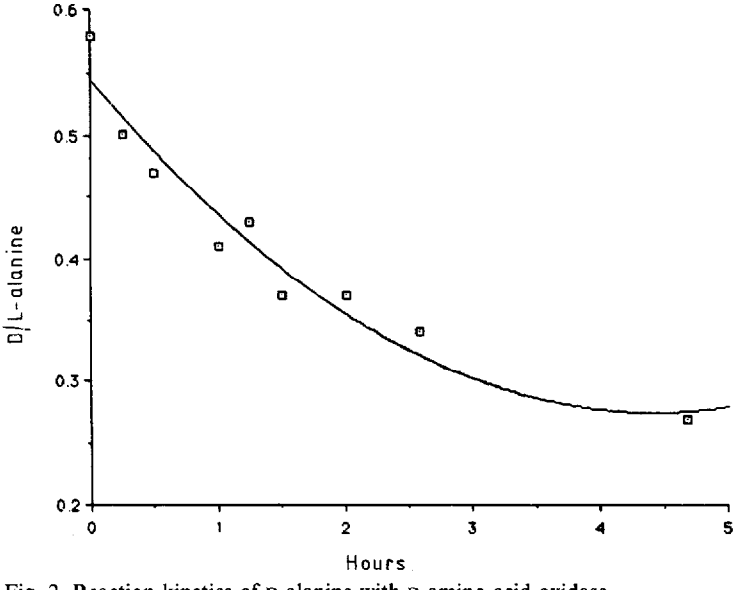

Fig. 2. Reaction kinetics of D-alanine with D-amino acid oxidase.

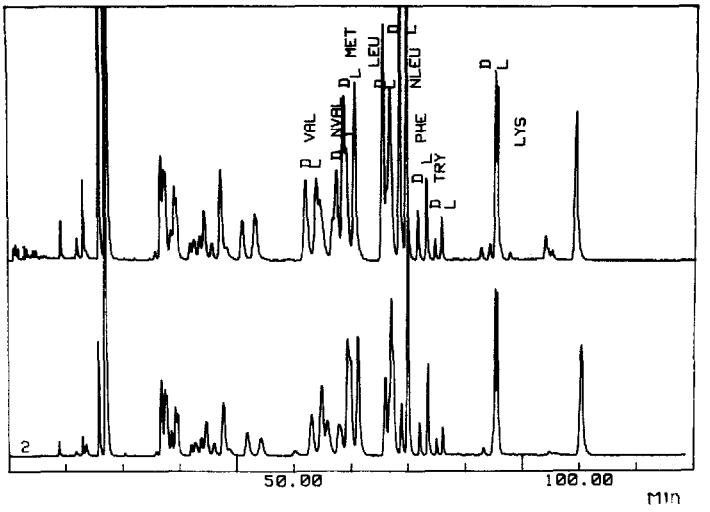

Fig. 3. Chromatogram of an amino acid mixture before (upper tracing) and after incubation with D-amino acid oxidase. Oxidative deamination of the D-isomers resulted in reduction in size of peaks corresponding to the D-amino acids. Mobile phase: 5.0 m*M* L-histidine methyl ester, 2.5 m*M* $CuSO_4 \cdot 5H_2O$ and 2.0 g of ammonium acetate, pH 5.5. A stepwise gradient was formed by blending the buffer with a 45% acetonitrile solution of the same buffer.

reached at equilibrium (Fig. 4). The same racemase reacted with either D- or L-alanine, giving a peak corresponding to the opposite isomer. After extended incubation, the same proportion of D- and L-isomers was obtained, regardless of whether the starting material was the D- or L-form (Fig. 5). There was no reaction of this racemase with serine, valine, leucine and methionine. Other microbacterial systems contain racemases similarly specific for other amino acids.

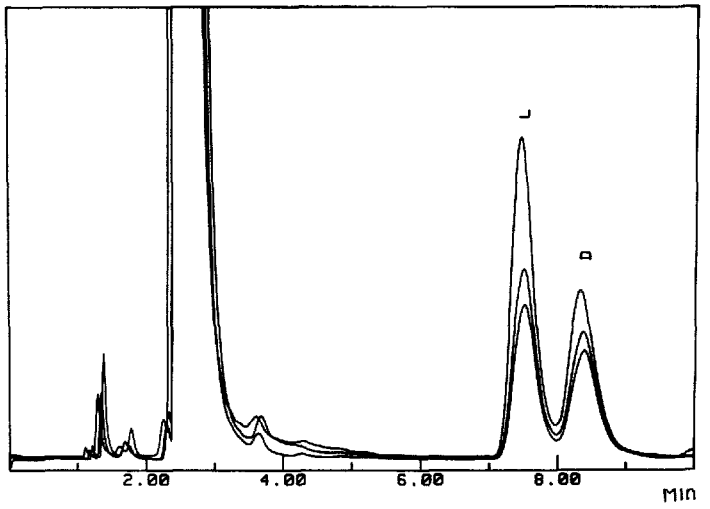

Fig. 4. Chromatogram of a racemic mixture of D,L-alanine incubated with cell extracts of *S. facecalis*. Constant D-to-L-alanine ratio was obtained at equilibrium (lower tracing). Conditions as in Fig. 1.

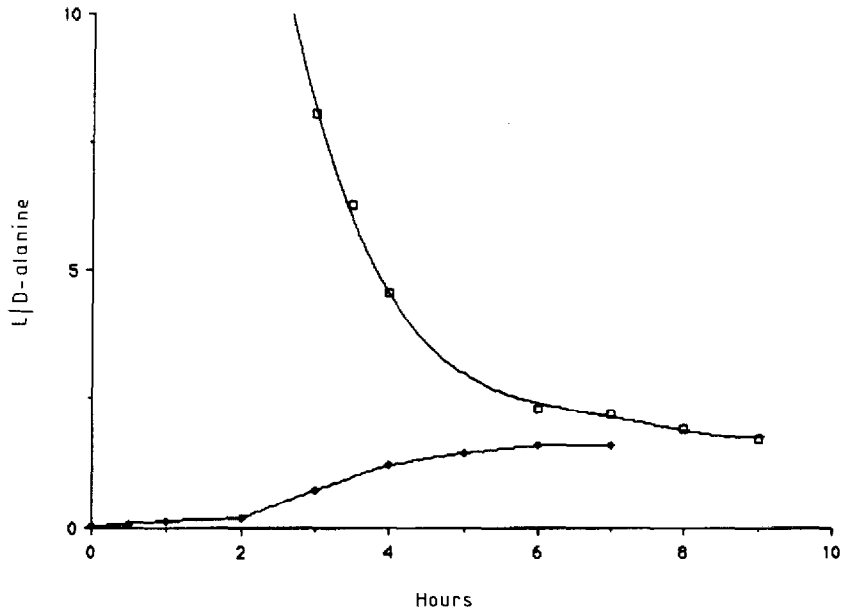

Fig. 5. Reaction kinetics of D- (◆) and L-alanine (□) with racemase.

DISCUSSION

D-Amino acid oxidase, used in the procedure described here, helped confirm the identification of D-amino acid isomers in chromatographic assays of complex mixtures. This enzyme is highly reactive with D-isomers and reacts with many different amino acids. For more specific identification of individual amino acids in a mixture, the racemase family of enzymes offers much greater specificity for individual amino acid pairs.

Different bacteria have racemases with different specificities toward amino acids. Chiral HPLC could obviously be used to characterize the racemases in cultures of individual bacterial species by incubating the bacteria with a mixture of L- or D-amino acids and then assaying the reaction mixture for the other isomers. We are not aware of anyone attempting to use the specificity of the racemases as a means of identifying or characterizing the bacteria.

We have also considered the use of D-amino acid oxidase in a post-column reactor for specific detection of the D-amino acids in a mixture. Highly sensitive enzymatic methods, based on specific oxidases, are widely used in clinical chemistry for assaying such metabolites as glucose, glycerol and cholesterol. For use in HPLC, reactors packed with an immobilized enzyme that is relatively resistant to denaturation by the mobile phase are generally preferred to adding soluble enzyme, if only for economic reasons. We have used post-column reactors containing semi-specific immobilized enzymes for highly specific and sensitive detection in HPLC of bile acids and steroid hormones[8]. For use with the system of amino acid HPLC described here, a post-column enzymatic detection method may require a method for reducing the

concentration of potentially toxic cupric ions in the HPLC effluent. This would be unnecessary if the HPLC separation of isomers were performed with a chiral stationary phase rather than with the copper complex method used here.

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