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Note

A post-column immobilized leucine dehydrogenase reactor for determination of branched chain amino acids by high-performance liquid chromatography with fluorescence detection

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Several cases of inborn errors of metabolism of branched chain amino acids (BCAAs) are known[1]. Usually, BCAAs are separated and quantitated by chromatographic procedures[2–8]. Post-column derivatization with ninhydrin, fluoresamine or *o*-phthalaldehyde is still the standard technique for amino acid analysis[9]. This paper describes a post-column reactor system for the high-performance liquid chromatographic (HPLC) determination of BCAA. The BCAAs from the separation column are mixed with a nicotinamide–adenine dinucleotide (NAD) solution and oxidized in a reactor containing immobilized leucine dehydrogenase (E.C. 1.4.1.9) (LDH). NAD is reduced to NADH according to the amount of BCAAs present in the solution. The NADH is monitored by fluorescence detection.

EXPERIMENTAL

Chemicals

Leucine dehydrogenase (38 U/mg of solid) was obtained from Toyobo (Osaka, Japan). Poly(vinyl alcohol) beads (9 μm, GS-520) were obtained from Asahi Kasei (Tokyo, Japan). NAD (grade II) was obtained from Boehringer Mannheim (Mannheim, F.R.G.). Capcell-C_{18} (10 μm) (Shiseido, Tokyo, Japan) and TSKgel SCX (5 μm) (Tosoh, Tokyo, Japan) were packed into stainless-steel columns of 250 mm × 4.6 mm and 300 mm × 7.8 mm, respectively, by the slurry-packing method. All other chemicals were of analytical grade.

For reversed-phase chromatography, the mobile phase was 25 mM sodium dihydrogenphosphate containing 0.5 mM Na$_4$EDTA. For ion-exchange chromatography, citrate buffer (pH 3.7) was used as the mobile phase and column temperature was kept at 60°C.

Immobilization of LDH

The method of epoxy activation and amination of the beads was similar to that of Matsumoto *et al.*[10]. The attached amine was measured by the Kjeldahl method[11] and amounted to 4.1 mequiv. per g of dry beads. The aminated beads were packed into a stainless-steel column (10 mm × 4 mm I.D.) by the dry-packing method. Glutaraldehyde solution (2.5%) in phosphate buffer (0.05 M, pH 7.0) was pumped
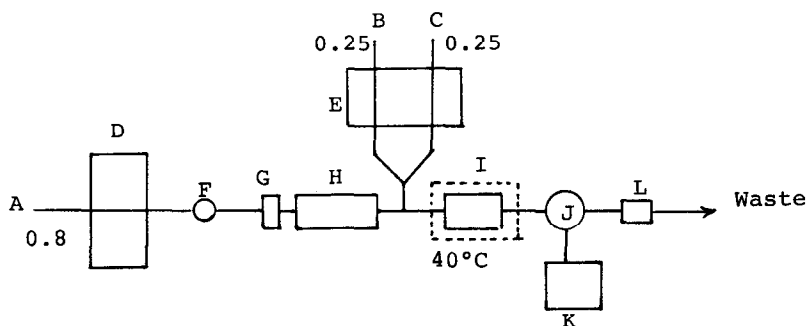
Fig. 1. An HPLC system for determination of valine, isoleucine and leucine with an immobilized leucine dehydrogenase reactor. A = Mobile phase (25 mM potassium dihydrogenphosphate + 0.5 mM Na$_4$EDTA); B = buffer (0.4 M glycine–potassium chloride/potassium hydroxide, pH 10.8 at 40°C); C = 35 mM NAD solution; D = HPLC pump; E = reagent pump; F = injector with 60-μl loop; G = precolumn (10 mm × 4.6 mm); H = analytical column (250 mm × 4.6 mm, Capcell-C$_{18}$); I = immobilized enzyme column reactor (10 mm × 4.0 mm); J = fluorescence spectrophotometer with a flow cell (18 μl); K = data processor; L = back pressure regulator. The column reactor was thermostatted at 40°C. The numbers given are flow-rates in ml/min.

through the column for 1 h at 0.1 ml/min and the column was washed with deaerated water for 20 min at 0.3 ml/min. The enzyme solution [5 mg in 10 ml of 0.05 M phosphate buffer (pH 7.0)] was circulated through the column at 0.1 ml/min for 3 h at room temperature. The enzyme solution was kept at about 4°C throughout the immobilization procedure.

HPLC system

The liquid chromatographic system is shown in Fig. 1. It consisted of a Bip 1 mobile phase pump (JASCO, Tokyo, Japan), a Rheodyne 7125 injector with 60-μl loop, a 1.0 cm × 4.6 mm I.D. stainless-steel precolumn, dry-packed with TSKgel ODS-120T (Tosoh), the separation column containing Capcell-C$_{18}$ (250 mm × 4.6 mm), a double plunger KHU-W-52 reagent pump (Kyowa Seimitsu, Tokyo, Japan), the column reactor packed with the immobilized enzyme, a 650 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) equipped with a flow cell (18 μl), a Chromatocorder II data processor (System Instruments, Tokyo, Japan) and a 02-0175 back pressure regulator (Chemco, Tokyo, Japan). The separation column and the column reactor were kept at ambient temperature and at 40°C, respectively.

The mobile phase, the NAD solution (35 mM) and the buffer 0.4 M glycine–potassium chloride/potassium hydroxide (pH 10.8 at 40°C) were pumped at 0.8, 0.25 and 0.25 ml/min, respectively, and mixed before the column reactor. Enzymatic reaction of Val, Ile and Leu proceeded in the reactor, and the NADH produced was monitored fluorimetrically at λ_{em} = 465 and λ_{ex} = 340 nm.

RESULTS AND DISCUSSION

Properties of immobilized LDH column reactor

The influence of pH on the reaction with Val, Ile and Leu was studied over the range between 10.0 and 11.5 using a glycine–potassium chloride/potassium hydroxide

(0.2 *M*) buffer. For this experiment, the guard and separation columns were omitted. Each standard (0.1 m*M*) was injected and mixed with NAD solution and buffer of various pH before the column reactor. As shown in Fig. 2, the optimum pH was about 10.8. Variation of the concentration of the buffer from 0.1 to 0.01 *M* did not affect the peak height. The peak height in the glycine buffer was about five times that in carbonate buffer at the same pH.

The peak height (at pH 10.8) increased with increasing temperature from 30 to 50°C, but at 55°C the enzyme was deactivated. For long-term usage, the reactor was thermostated at 40°C.

Since the Michaelis constants of the immobilized LDH for Leu and NAD were 1.3 and 0.50 m*M*, respectively, under the conditions of Leu < 0.1 m*M* and NAD > 5 m*M*, the kinetics of the reaction was first order and zero order, respectively. The concentration of NAD in the reactor was kept at 6 m*M*. The peak height for 0.01 m*M* Leu decreased linearly with increasing flow-rate from 0.5 to 1.5 ml/min.

D Isomers of BCAAs, Co^{2+} and Cu^{2+} inhibited the enzymatic reaction. Interference from the metal ions was avoided by addition of EDTA to the flow stream. The peak height of the L isomer (0.01 m*M*) was decreased by 10 and 50% in the presence of 0.01 and 0.02 m*M* of the D isomer, respectively.

As measured with 0.01 m*M* BCAAs and 6 m*M* NAD in glycine–potassium chloride/potassium hydroxide buffer (pH 10.8) at a flow-rate of 1.3 ml/min, the relative activities of the immobilized LDH for Leu, Ile and Val were 100, 82 and 72, respectively. On the other hand, in the free enzyme, the values were 100, 55 and 74,
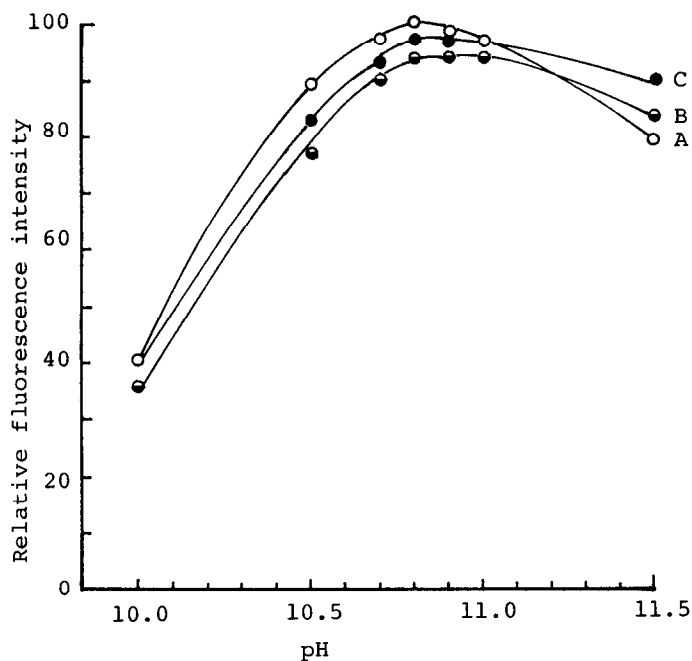

Fig. 2. Effect of pH on the activity of immobilized leucine dehydrogenase. A, L-leucine; B, L-valine; C, L-isoleucine.
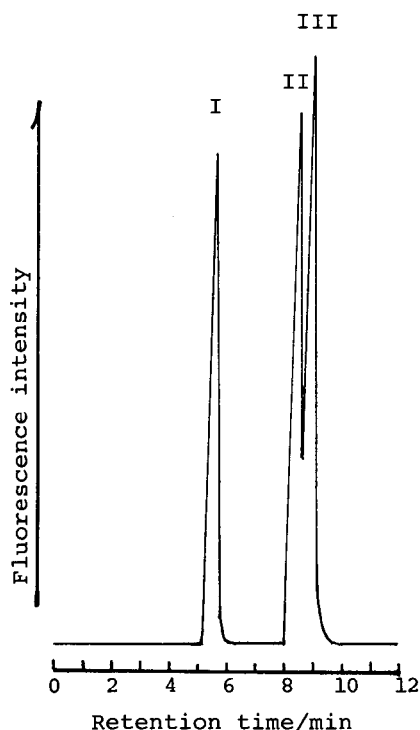
Fig. 3. Chromatogram of a standard solution (0.01 m*M* of each compound). Peaks: I = L-valine; II = L-isoleucine; III = L-leucine.

respectively. The free enzyme was examined by using an enzyme solution (7 U/ml) prepared by dissolving LDH in the NAD solution (6 m*M*) and a reaction tube (PTFE, 100 mm × 0.5 mm I.D.) instead of the reactor. Many examples of changed specificities of immobilized enzymes relative to free enzymes are known[12]. The change in specificity of LDH immobilized on the polymer beads may be attributed to steric interference caused by covalent attachment.

To confirm the long-term stability of the reactor, it was used for 6 h in a day and stored at 4°C in 0.1 *M* phosphate buffer containing 1 m*M* Na$_4$EDTA when not in use. The enzyme retained more than 60% of its original activity after 1 month.

TABLE I

SEPARATION PARAMETERS FOR BRANCHED CHAIN AMINO ACIDS

Amino acid	Retention time (min)	Capacity factor	Resolution	Separation factor
L-Valine	5.5	0.37		
			8.16	2.7
L-Isoleucine	8.3	1.0		
			0.71	1.2
L-Leucine	8.8	1.2		

TABLE II

RESULTS FOR FREE BRANCHED CHAIN AMINO ACIDS IN CONTROL SERA

Serum	Amino acids found (μM)*					
	Proposed method			o-Phthalaldehyde method		
	Val	Ile	Leu	Val	Ile	Leu
Serum I**	233	76	124	235	79	130
Serum II***	220	61	119	221	66	121

  * $n = 5$.
 ** Precinorm U, No. 155 657, Boehringer Mannheim.
*** Precinorm U, No. 155 171, Boehringer Mannheim.

Separation of BCAAs

A combination of separation of BCAAs by chromatography and the enzymatic reaction was attempted. In reversed-phase ion-pair chromatography, the immobilized LDH was unstable when allowed to stand in contact with ionic surfactants for a long time. In ion-exchange chromatography, the resolution of BCAAs was inferior to that in bonded phase chromatography. Separation of BCAAs was effected on a Capcell-C_{18} column with 25 mM sodium dihydrogenphosphate containing 0.5 mM Na_4EDTA as the mobile phase, as shown in Fig. 3. The separation parameters for the BCAAs are listed in Table I. The ratio of peak heights for the same concentration of Leu, Ile and Val was 100:90:83.

The peak heights were plotted against the concentrations of the amino acids. Four calibration graphs were prepared for Leu, Ile and Val, covering the ranges of 1–5, 5–10, 10–50 and 50–100 μM. The detection limit was 0.5 μM.

Application

The method was applied to the determination of free BCAAs in control serum. The serum (0.1 ml) was deproteinized by adding 0.8 ml of 1/6 M sulphuric acid and 0.1 ml of 10% sodium tungstate. The suspension was filtered through a column gurd filter (pore size 0.45 μm). An aliquot (60 μl) of the filtrate was injected into the column. The results obtained by the present method and by ion-exchange chromatography on the basis of post-column derivatization with o-phthalaldehyde–2-mercaptoethanol are shown in Table II.

In conclusion, this method proved to be simple for the determination of free branched chain amino acids, since only the separation of the three amino acids from each other is required for the assay. The method may easily be used routinely to determine BCAAs.

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