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Note

Assay for lysine-ketoglutarate reductase by reversed-phase high-performance liquid chromatography

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Lysine is an essential amino acid in the diet of animals, including the human. The two major pathways in lysine catabolism involve the formation of either saccharopine or pipecolic acid [1–3]. Previous studies in the rat, chick, and human have indicated that the saccharopine pathway is the major route of lysine breakdown [4–6]. The enzyme catalyzing the conversion of L-lysine to saccharopine is lysine-ketoglutarate reductase (EC 1.5.1.8). The enzyme requires α-ketoglutarate and NADPH as cofactors and catalyzes a reversible reaction.

Fjellstedt and Robinson [7] measured lysine-ketoglutarate reductase activity by determining the rate of oxidation of NADPH at 340 nm on a recording spectrophotometer. Other authors [8,9] have measured the end product, saccharopine, via amino acid analysis of the derivatized amino acid, and used this to calculate the enzyme activity. The current paper describes a new technique for determining saccharopine for the purpose of measuring lysine-ketoglutarate reductase activity, utilizing reversed-phase high-performance liquid chromatography (HPLC) and *o*-phthalaldehyde (OPA) pre-column derivatization.

EXPERIMENTAL

Reagents

Methanol (HPLC grade) was obtained from Burdick and Jackson (Baxter Healthcare Corporation, Romulus, MI, U.S.A.). Mercaptoethanol, α-amino-

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β-guanidinopropionic acid (AGPA) and the OPA (sold as fluoraldehyde) were obtained from Pierce (Rockford, IL, U.S.A.). Saccharopine, NADPH, α-ketoglutarate, and L-lysine were obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical-reagent grade from commercial sources.

Sample preparation

Pregnant Sprague–Dawley rats (Charles River, Portage, MI, U.S.A.) were fed a crystalline amino acid diet for three weeks. The animals were killed at day 20 of gestation, their livers removed and quickly frozen via aluminum clamps cooled in liquid nitrogen. The tissues were held at −30°C until analysis. The tissues were homogenized in 0.1 *M* phosphate buffer, pH 7.4 (1 g tissue per 4 ml buffer). Homogenate protein concentrations were measured by the method of Lowry et al. [10].

Assay

The assay was that of Wang and Nesheim [11], as modified by Scott and Austic [9]. Each sample was analyzed in triplicate, with one of the three replicates being the blank. In the blank samples, the lysine was added after the reaction was terminated. The assay mixture contained 2 m*M* NADPH, 3 m*M* L-lysine, 5 m*M* α-ketoglutarate, and 0.5 ml homogenate in 0.9 ml. Upon addition of the L-lysine, the tubes were flushed with nitrogen, sealed and incubated for 30 min at 37°C. The reaction was terminated by the addition of 0.25 ml of 12% perchloric acid. To each tube was then added 0.1 ml of 1.25 m*M* AGPA (the internal standard). The tubes were allowed to stand on ice for 30 min and then centrifuged at 1500 *g* for 10 min. The supernatant was transferred to another tube containing 0.25 ml of cold 2 *M* potassium bicarbonate, vortex-mixed, placed on ice for 30 min, and centrifuged at 1500 *g* for 10 min. The supernatants were then applied to Microfilteruge tubes (0.45 μm, Rainin Instruments, Woburn, MA, U.S.A.), and centrifuged at 750 *g* for 10 min. A 50-μl aliquot of the sample was transferred to a 32 mm×12 mm polypropylene autosampler vial (200-μl volume, Sun Brokers, Wilmington, NC, U.S.A.), and the vial was capped with a septum and cap.

The standards were prepared by mixing saccharopine and AGPA. The limit of detection (signal-to-noise ratio 4:1) was 400 pmol saccharopine injected on the column. Standard concentrations of saccharopine ranged from 31.25 to 250 μ*M*. A 50-μl aliquot of each in duplicate were added to autosampler vials as described above. To the autosampler were added 50 μl of an OPA mixture (50 mg of OPA, 1.25 ml of methanol, 11.25 ml of 0.4 *M* sodium borate, pH 9.5, and 50 μl of mercaptoethanol). The solution was mixed and allowed to react for 1 min, and then 50 μl were injected onto the chromatographic system.

Chromatography

The HPLC system consisted of two Model 110A pumps, a 3-μm Ultrasphere-XL ODS column (70 mm × 4.6 mm) and guard column (5 mm × 4.6 mm; Beckman Instruments, Fullerton, CA, U.S.A.), an LC-4B amperometric detector, an RE-1 reference electrode, and a glassy carbon working electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a KORTEC K65B automated sample injector (Rainin Instruments), and a Maxima 820 chromatography workstation (Millipore, Milford, MA, U.S.A.). The workstation utilized a Leading Edge Model D computer (Leading Edge Products, Canton, MA, U.S.A.) and a Data Translation DT2805 data acquisition board (Data Translation, Marlborough, MA, U.S.A.) to control the gradient, acquire the data from the electrochemical detector, and determine peak size.

The two mobile phases were 0.1 *M* sodium acetate, pH 6.7 (reagent A) and methanol (reagent B). The gradient used for the assay was as follows: 0–0.5 min, linear increase from 16 to 20% methanol; 11.5–12.0 min, linear increase to 80% methanol; 19.0–19.5 min, linear decrease to 16% methanol. The column was re-equilibrated for 8.5 min prior to the next injection. The flow-rate was 1.6 ml/min. The applied electrical potential was +0.500 V.

RESULTS AND DISCUSSION

The chromatographic profiles showing saccharopine and AGPA are shown in Fig. 1. Fig. 1A shows a blank from rat liver for the reaction. Peak 2 is the internal standard, AGPA. Fig. 1B shows a chromatogram from a lysine-ketoglutarate reductase assay from rat liver, in which peak 1 is saccharopine and peak 2 is AGPA. The product was identified by co-chromatographing with a commercially available saccharopine.

Standard curves on four successive days showed a linear relationship between concentration of saccharopine (μM) and the area ratio of saccharopine to AGPA, with a mean r^2 for four analyses of 0.972. Slopes of these four replicate standard curves had a coefficient of variation (C.V.) of 12.4%. The C.V. of ten successive injections of a standard was 4.2%, while the C.V. of four successive determinations from a rat liver was 8.1%. The C.V. values for four successive days from two rat livers, in terms of enzyme activity, were 9.7 and 4.3%. The hepatic lysine-ketoglutarate activity for the rats was 6.45 ± 0.71 mol/g/h, or 59.78 ± 11.23 nmol/h when expressed per mg of protein (mean ± S.D.). In the study of Chu and Hegsted [8], lysine-ketoglutarate reductase activities (mean ± S.E.M.) of 10.81 ± 1.82 μmol saccharopine per g liver per h were reported in rats fed a crystalline amino acid diet. Scott and Austic [9] reported a value of 9.1 ± 0.3 μmol saccharopine per g liver per h in chicks fed a casein-based diet. The rats in this study were pregnant and fed a crystalline amino acid diet, which may account for the discrepancies between the values.

The HPLC lysine-ketoglutarate reductase assay described herein requires

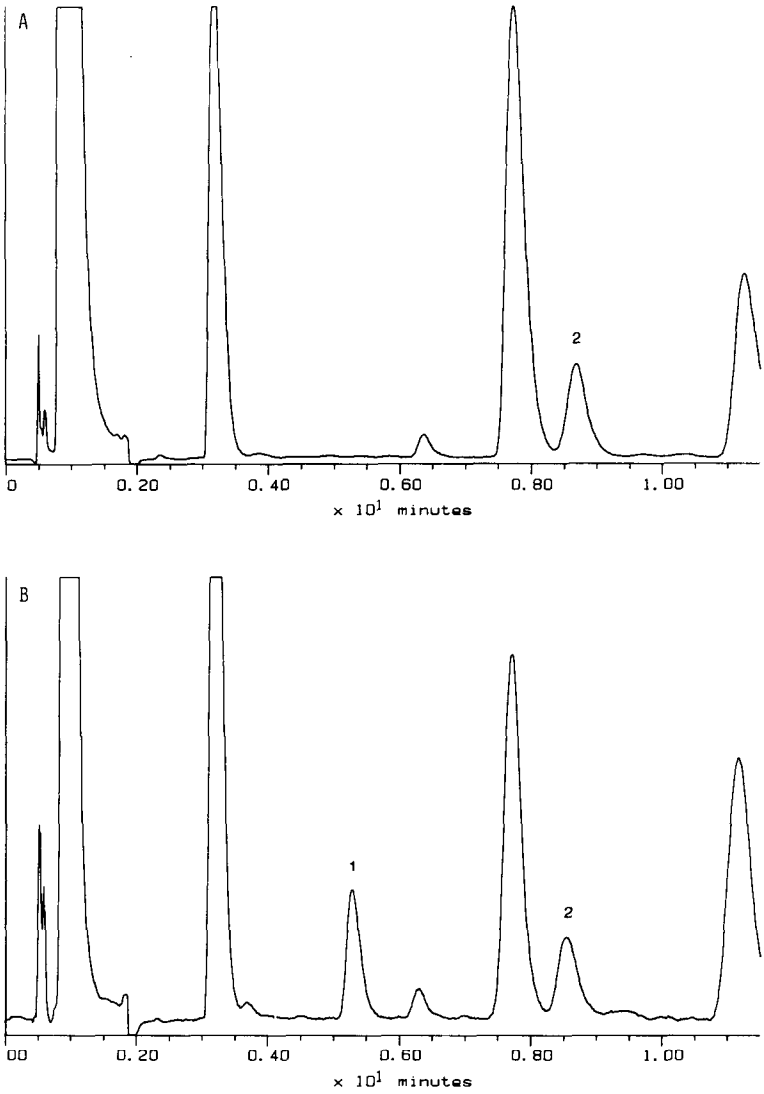

Fig. 1. Chromatogram of hepatic lysine-ketoglutarate reductase assay. (A) Blank, i.e., lysine added after deproteinization of the enzyme; (B) lysine added to start the reaction. Peaks: 1 = saccharopine; 2 = AGPA. Chromatographic conditions: 3-μm Ultrasphere-XL ODS column (70 mm × 4.6 mm); gradient elution, 0.1 *M* sodium acetate (pH 6.7) and methanol (gradient described in text); flow-rate, 1.6 ml/min. The electrochemical detector was operated with an applied electrical potential of +0.500 V, using a glassy carbon working electrode. The *y*-axis represents the voltage signal acquired from the electrochemical detector.

27 min between samples, with 19 min to run the assay and 8.5 min to re-equilibrate the column. The assay reported previously, which was based on the separation and determination of saccharopine, has been run using a Technicon TSM amino acid analyzer [8,9]. The present study, using the internal standard AGPA, has successfully utilized the technique of HPLC to make a similar separation and determination.

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