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Chemistry & Drug Discovery

Supporting Information

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Tissue

Animals were killed with an overdose of chloral hydrate (500 mg/kg), and their livers were immediately dissected out and frozen on dry ice.

Partial enzyme purification

Approximately 20 g of tissue were thawed and homogenized (1:5, w/v) in 2.5 mM Buffer A (phosphate buffer at pH 7.4 containing 10 mM 2-mercaptoethanol) containing 50 µM pyridoxal-5'-phosphate using a Polytron (Kinematica, Luzern, Switzerland). The homogenate was then sonicated for 5 min (Branson, Danbury, CT, USA, setting 6). After centrifugation (10,000 x g, 10 min), the resulting supernatant was applied to a DEAE-Sepharose column (5 x 9 cm) equilibrated with 2.5 mM buffer A. The column was washed with an equal volume of 2.5 mM buffer A, and proteins were eluted with a linear gradient of 500 ml of 2.5-100 mM buffer A followed by 50 ml of 100 mM buffer A and 200 ml of 250 mM buffer A. Active fractions were combined and concentrated by ultrafiltration under nitrogen using a cellulose membrane (YM 10, Millipore Corp., Bedford, MA, USA). The enzymes were diluted with 1 M buffer A to obtain a final concentration of 500 mM and subsequently applied to a Phenyl-Sepharose column (3.5 x 5 cm), equilibrated with 500 mM buffer A. Proteins were eluted by a linear gradient of 300 ml of 500-2.5 mM buffer A plus 150 ml of 2.5 mM buffer A. Active fractions were combined and concentrated by ultrafiltration as described above. Partially purified

preparations of KAT I and KAT II were then concentrated using a Centriplus YM 10 membrane (Millipore Corp., Billerica, MA, USA).

KAT assay

KAT activity was assayed by using a slightly modified procedure developed by Schmidt at al. (1993). Briefly, the reaction mixture contained the enzyme preparation, ^{3}H-kynurenine (final concentration: 2 µM), 1 mM pyruvate, 70 µM pyridoxal-5'-phosphate and 150 mM Tris-acetate (pH 7.4) in a total volume of 200 µl. Test compounds were added in 20 µl aliquots immediately before initiating the assay. Blanks were prepared by boiling the homogenate for 10 min. The samples were incubated for 2 hours at 37°C, and the reaction was terminated by the addition of 20 µl of 50 % (w/w) trichloroacetic acid and 1 ml of 0.1 M HCl. Denatured proteins were removed by centrifugation, and newly synthesized ^{3}H-KYNA was recovered from Dowex 50W cation exchange columns and quantified by liquid scintillation spectrometry.

Kynurenine 3-hydroxylase assay

Kynurenine 3-hydroxylase activity was determined according to the method of Erickson et al. (1992) using [3,5-^{3}H]-kynurenine (final concentration: 2 µM) as a substrate.

Kynureninase assay

Kynureninase activity was measured by quantifying the amount of ^{3}H-anthranilic acid formed from the catalytic transformation of ^{3}H-kynurenine. The reaction mixture

contained the enzyme preparation (1:300, w/v), ^{3}H-kynurenine (final concentration: 2 µM), 2.5 µM pyridoxal-5'-phosphate, 5 mM 2-mercaptoethanol and 92.5 mM Tris-HCl (pH 8.4) in a volume of 200 µl. Test compounds were added in 20 µl aliquots immediately before initiating the assay. Blanks were prepared by boiling the homogenate for 10 min. The samples were incubated for 2 hours at 37°C, and the reaction was terminated by the addition of 50 µl of 6 % perchloric acid. Newly formed ^{3}H-anthranilic acid was separated by HPLC (mobile phase: 80 mM monobasic potassium phosphate:acetonitrile:methanol, 75:15:10; flow rate: 1 ml/min) and quantified by liquid scintillation spectrometry. The retention time of anthranilic acid in this system was ~9.7 min.

References

J.B. Erickson, E.M. Flanagan, S. Russo, J.F. Reinhard Jr. *Anal Biochem.* **1992,** *205,* 257-62.

W. Schmidt, P. Guidetti, E. Okuno, R. Schwarcz. *Neuroscience* **1993,** *55,* 177-84.