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Note

Assay of γ-L-glutamylcyclotransferase activity in rat brain synaptosomes by high-performance liquid chromatography

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γ-L-Glutamylcyclotransferase (EC 2.3.2.4) catalyses, in different animal tissues, the conversion of some γ-glutamylamino acids into 5-oxo-L-proline and free amino acid.

5-Oxo-L-proline was generally regarded as a cyclic product that originates non-enzymatically from glutamate or glutamate derivatives. Connell and Hanes [1] first demonstrated the enzymatic formation of such a product from γ-glutamylglycine, γ-glutamylglutamate and, to a lesser extent, γ-glutamylphenylalanine. Nowadays, 5-oxo-L-proline is considered as an active metabolite and an intermediate in the γ-glutamyl cycle [2].

Intracellular γ-L-glutamylcyclotransferase [3] and the membrane-bound γ-glutamyltranspeptidase [4] are the key enzymes for the coupling of the amino acid uptake to the glutathione metabolism [5]. Relatively high levels of γ-glutamylcyclotransferase activity have been found in kidney, liver, brain and skin, with significant differences in different species [6], and highly purified enzyme preparations have been obtained from human and sheep brain [7].

It is of interest that significant amounts of 5-oxo-L-proline are present in both the cerebrospinal fluid (CSF) and brain tissue, and that the CSF levels of this metabolite are higher than the plasma level [8]. The CSF concentration of 5-oxo-L-proline is about six times higher than that of the serum in patients with 5-oxoprolinuria [9].

There is some evidence that the enzymes of the γ-glutamyl cycle are present in the endothelium of brain capillaries and in the choroid plexus [10], a structure involved in the secretion of CSF and in the maintenance of brain homeostasis.

The γ-glutamyl cycle may operate in the transport of amino acids from the

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ventricular CSF to the blood and, in particular, γ-glutamylcyclotransferase is more active in the choroid plexus than in other brain regions [11].

Despite the wide distribution in the brain of γ-glutamyl derivatives, such as glutamine, glutamate, glycine and several other amino acids, little is known about the role and localization of γ-glutamylcyclotransferase in neurons, subcellular fractions or glial cells, and only some histochemical studies have been conducted on the localization of the correlated enzyme, γ-glutamyltranspeptidase, in perikarya of specific neurons within the brain stem [12].

This paper reports a new and sensitive method for the measurement of the γ-glutamylcyclotransferase activity of rat brain synaptosomes, based on the detection of 5-oxo-L-proline by reversed-phase high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Reagents and chemicals

Ammonium phosphate monobasic crystalline HPLC reagent (Baker, Phillipsburg, NJ, U.S.A.), 5-oxo-L-proline (Ega-Chemie, Steinheim, F.R.G.), γ-glutamylglycine and γ-L-glutamylglutamine (Sigma, St. Louis, MO, U.S.A.) and Ficoll 400 (Pharmacia, Uppsala, Sweden) were used.

Chromatographic system

The determinations were carried out with a Perkin-Elmer chromatographic system (Perkin-Elmer, Norwalk, CT, U.S.A.) consisting of a Series 3B liquid chromatograph, an LC 75 spectrophotometric detector operated at 212 nm, an LC1 100 laboratory computing integrator set at attenuation 16, a C_{18} SIL-X-10 column (250 mm × 4.6 mm I.D.; 10 μm average particle size), an RP-8 Spheri-5 OS-GU guard column (Brownlee Labs., Santa Clara, CA, U.S.A.). The eluent was a 0.025 *M* ammonium phosphate buffer (pH 3.5), and the separations were carried out at a constant flow-rate of 1 ml/min at 25 °C.

Under these conditions, the retention times of the test substances were 3.90 min for γ-L-glutamylglycine, 4.16 min for γ-L-glutamylglutamine and 7.12 min for 5-oxo-L-proline.

Synaptosomes isolation

In a typical experiment, the cerebral cortex from two rat brains was suspended in an ice-cold isolation medium of 0.32 *M* sucrose, 0.005 *M* N-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid and 0.5 m*M* EDTA at pH 7.4. The cortex was chopped and gently homogenized in a glass homogenizer equipped with a Teflon pestle (ca. twelve strokes). Synaptosomes were isolated from a discontinuous Ficoll gradient of 4 ml of 12%, 1 ml of 9% and 4 ml of 6% Ficoll in the isolation medium, according to Nicholls [13]. The gradient was centrifuged with a swing-out rotor in a Beckman L3-50 ultracentrifuge, at 75 000 *g* for 30 min; alternatively an angle rotor (42.1 at 31 000 rpm per 30 min) was used.

The purity of the synaptosomal fraction was checked by electron microscopy

(data not shown). Synaptosomes were layered in two narrow bands at the 9% Ficoll interface. After removal the fraction was washed with five volumes of the isolation medium and centrifuged at 27 000 *g* in a Sorvall Superspeed RC2-B centrifuge for 10 min. The pellet was resuspended in 2 ml of the isolation medium, and protein was determined by the Biuret method [14].

Determination of enzyme activity

Synaptosomes were lysed with ten volumes of bidistilled water buffered with 0.005 *M* Tris–HCl at pH 8.6 [15]. The suspension, containing ca. 2 mg/ml of protein, was homogenized with a glass pestle and allowed to stand in ice for 1 h.

Lysed suspension (0.1 ml) was added to 0.1 ml of a mixture of Tris–HCl at a final concentration of 0.08 *M* (pH 8.0), and the substrate at a final concentration of 0.002 *M*. Incubation was carried out in Eppendorf test-tubes at 37 °C from 30 min to 2 h, within the time linearity range [7].

The reaction was stopped by denaturation at 96 °C for 2 min, the tubes were centrifuged, and a constant injection volume of 10 μl of the supernatant was used. The substrate was added 10 s before heat denaturation in blank tubes incubated at 37 °C for the same time intervals.

RESULTS AND DISCUSSION

A calibration curve for 5-oxo-L-proline was set up by injecting 10 μl of different dilution samples, from 0.05 to 2.5 m*M*, suspended in the same elution buffer. There was a linear relationship between the amount of 5-oxo-L-proline injected and the peak areas: the linear regression equation ($r=0.97, p<0.01$) was $y=0.06x$, where y is the peak area ($\times 10^6$) and x the amount of 5-oxo-L-proline, in nmol. The precision of the method was determined: the coefficient of variation (C.V.) was calculated, from day-to-day measurements of the synaptosomal activity, to be 1.35–2.75%. The γ-L-glutamylcyclotransferase activity, measured in a lysed synaptosomal fraction without further purification, was linear ($r=0.98, p<0.01$) with amounts of synaptosomal protein between 0.05 and 0.25 mg/ml.

The optimal substrate concentration was 0.002 *M* with γ-L-glutamylglutamine and 0.005 *M* with γ-L-glutamylglycine, which are considered as the best natural substrates [7].

A typical elution pattern, with γ-L-glutamylglutamine as substrate, is shown in Fig. 1. The specific activities, with different substrates, are listed in Table I. The identity of the reaction product was checked by adding the standard to the sample (Fig. 1C).

Other methods [7, 16] are currently used for the determination of the γ-glutamylcyclotransferase activity. One is based on the fact that the *p*-nitroanilide derivative of the amino acid is hydrolysed more rapidly in alkali than the *p*-nitroanilide of the dipeptide used as a substrate. Another method is based on the absorbance of 5-oxo-L-proline, detected after eluting the reaction mixture from Dowex 50 (H^+) columns [6], but it seems that there is no generally accepted reference method for the measurement of the activity of this enzyme.

The procedure described in this paper represents an improvement over the

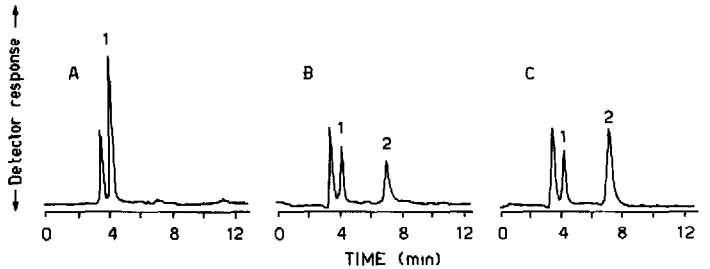

Fig. 1. Determination of the enzymatic production of 5-oxo-L-proline by rat brain synaptosomes (0.2 mg of protein) after incubation with γ-L-glutamylglutamate at a final concentration of 0.002 *M* for 2 h at 37°C (see Experimental). (A) A 2-h blank; (B) a 2-h sample; (C) a 2-h sample spiked with 3 nmol of 5-oxo-L-proline. Peaks: 1 = γ-L-glutamylglutamine (retention time 4.16 min); 2 = 5-oxo-L-proline (7.12 min).

TABLE I

SPECIFIC ACTIVITY OF γ-L-GLUTAMYLCYCLOTRANSFERASE WITH TWO DIFFERENT SUBSTRATES IN RAT BRAIN SYNAPTOSOMES

For details see Experimental; mean values of six determinations ± S.D.

Substrate	Activity (nmol mg^{-1} protein h^{-1})
γ-L-Glutamylglutamine (0.002 *M*)	177.2 ± 2.4
γ-L-Glutamylglycine (0.005 *M*)	112.6 ± 3.1

existing procedures since its sensitivity is at least 40-fold higher, allowing the detection of 0.5 nmol of 5-oxo-L-proline. The method is rapid, precise and suitable for the study of the parallel disappearance of the substrate and the effect of drugs separable from 5-oxo-L-proline on the basis of the different elution times.

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