MICELLAR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FOLYLPOLYGLUTAMATE HYDROLASE ACTIVITY

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SUMMARY

   A rapid and sensitive method for the quantitative determination of folylpolyglutamate hydrolase activity in crude tissue extracts was developed. The procedure is based on high-performance liquid chromatographic separation of folate analogue mono- and polyglutamates on a reversed-phase column using sodium dodecyl sulfate in water as the mobile phase. Interfering substances in tissue extracts were removed by gel filtration on centrifugally-eluted mini-columns of Sephadex G-25 prior to incubation of polyglutamate substrate with tissue extract hydrolase. Reactions were terminated by denaturation of the enzyme in sodium dodecyl sulfate, which subsequently served as the micellar solvent system for chromatographic separation of substrate from reaction products.

INTRODUCTION

   Folylpolyglutamate hydrolase specifically catalyzes the hydrolytic cleavage of peptide bonds involving the γ-carboxyl group of glutamic acid. The enzyme is relatively nonspecific with regard to the pteridine or pteridine-like moiety. This enzyme activity has been observed in numerous tissues from microorganisms, plants and animals[1] and may be of lysosomal and/or cytosolic origin. Both endo- and exopeptidase activities have been reported[1]. Since folates in food occur mainly as polyglutamates which are poorly absorbed, much of the interest in these enzymes has centered on their role in the bioavailability of dietary folates[2–8]. However, they may also play a role intracellularly in the control of one-carbon metabolism. Intracellular folates are almost entirely polyglutamyl derivatives and changes in polyglutamate chain lengths are known to have dramatic effects on the kinetics of a number of folate-requiring enzymes[9–11]. Studies of the role, if any, of the hydrolase in the regulation

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of intracellular polyglutamate chain lengths have been hampered by the lack of a convenient assay.

Early microbiological assays of folylpolyglutamate hydrolase[12,13] were sensitive, but also lengthy, hard to reproduce, subject to interference and lacked specificity. These procedures have been improved by Elsenhaus *et al.* through the use of short term uptake of radiolabelled products by microorganisms[14], but specificity is still lacking. These workers have also used paper chromatographic[2] and high voltage paper electrophoretic[5] techniques for qualitative and quantitative assessment of the products of enzymatic hydrolysis in purified or highly active enzyme preparations.

A rapid and sensitive radioassay was developed by Krumdieck and Baugh[15] and modified by Silink *et al.*[16]. However, preparation of the required labelled substrates with high specific activity is difficult and expensive. We have previously described[17] a procedure involving polyacrylamide gel electrophoretic separation of ternary complexes of methylenetetrahydrofolate polyglutamates with thymidylate synthase and commercially-available tritiated-fluorodeoxyuridylate of high specific activity. Although the method is highly sensitive, applicable to crude extracts and allows specific product identification, it is not generally useful for monitoring hydrolase activity (*e.g.* during purification procedures) because of the analysis time required. Recently, a number of rapid high-performance liquid chromatographic (HPLC) separations of folate and folate-analogue polyglutamates have been reported[18-25] and reversed-phase HPLC on octadecylsilica (C_{18}) has been used to assay the hydrolase activity in purified preparations from human intestinal mucosal brush border and hog kidney[26].

We report here a simple and rapid micellar HPLC procedure for the assay of folylpolyglutamate hydrolase activity based on direct injection of the reaction mixture following quenching with sodium dodecyl sulfate (SDS). The substrate chosen for this procedure was 5,8-dideazaisopteroyl-γ-glutamyl-L-glutamic acid (IAHQ-Glu, I, $n = 1$) because of its relative ease of preparation and availability[27]. However, the corresponding derivative of methotrexate, MTX-Glu, is also suitable for use in this assay.

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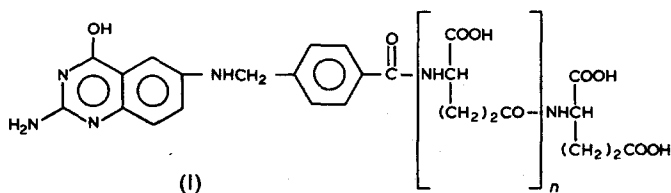
Separation of substrate and product(s) is accomplished with a mobile phase of SDS in water using reversed-phase HPLC on a C_{18} column under isocratic conditions. Interfering substances in crude extracts are removed prior to hydrolase assay by chromatography on mini-columns of Sephadex G-25.

EXPERIMENTAL

Reagents

SDS, Puriss grade, was obtained from Fluka and used as received. Mobile

phase for HPLC separations consisted of 0.2 M SDS in distilled, deionized water filtered through a 0.45-μm pore size nylon filter membrane from Alltech. Methotrexate (MTX) was purchased from Lederle and the triglutamate (MTX-Glu$_2$) was obtained from the National Cancer Institute. IAHQ and its polyglutamates, IAHQ-Glu and IAHQ-Glu$_2$, were prepared as previously reported[27,28]. Aqueous solutions of these folate analogues (1 mg/ml) were stable indefinitely at $-20°$C. However, the IAHQ compounds were stable for only three days at room temperature and one month at 4°C. All other chemicals were of reagent grade.

Enzyme preparations

Crude extracts of folylpolyglutamate hydrolase were prepared from kidneys of male CBA mice obtained from the Department of Lab Animal Medicine, Medical University of South Carolina. Tissues were homogenized in 9 volumes of ice cold 0.05 M acetate buffer, pH 4.4, and allowed to autolyse at 37°C for 30 min to hydrolyze endogenous folate polyglutamates. Autolyzed extracts were centrifuged at 32 500 g for 20 min prior to gel filtration to remove low-molecular-weight compounds which interfere with HPLC analysis.

Gel filtration through Sephadex G-25 was carried out in mini-columns made from 35 × 8 mm I.D. cylindrical plastic centrifuge tubes punctured at the tip with a syringe needle and supported in a plastic counting minivial. Columns of this type had a bed volume of *ca.* 1.5–2 ml. Excess buffer was removed by centrifugation at 1000 g for 6 min in a table top centrifuge. Samples of 150–200 μl were applied to columns and recovered with no significant loss or dilution following centrifugation through the gel at 1000 g for 6 min. The filtrate obtained was used as a source of enzyme without further treatment.

Enzyme assay

Stock solutions (1 mg/ml) of substrate were mixed with crude tissue extract (treated as described above) usually in a ratio of 1 volume of substrate to 19 volumes of tissue extract and incubated for 60 min at 37°C. This gave a substrate concentration of *ca.* 85 μM in the reaction mixture. The reaction was stopped by addition of 1 M SDS in an amount equal to 25% of the reaction volume.

Equipment

Isocratic reversed-phase HPLC was carried out using a Perkin-Elmer Series 2 liquid chromatograph equipped with a Whatman PXS 10/25 ODS column and Rheodyne injection valve. Absorbance at 254 nm was measured using a Perkin-Elmer LC55 spectrophotometer with a 8 μl flow cell. A Perkin-Elmer 3600 data station with Chromatographics 2 software was used for data presentation and analysis.

HPLC

Isocratic HPLC was carried out at a constant flow-rate of 1.0 ml/min using 0.2 M SDS in water as the mobile phase. Preliminary studies with solutions ranging from 0.05 M to 0.6 M SDS indicated that while 0.6 M SDS mobile phase gave slightly longer retention times, 0.2 M gave better separation of substrate and product. A typical analysis consisted of the direct injection of a 50-μl aliquot from a reaction mixture that had been quenched by the addition of 0.25 volume of 1 M SDS.

RESULTS AND DISCUSSION

The present study was prompted by a need for a simple, rapid assay for fo-lylpolyglutamate hydrolase activity that could be applied to crude extracts of biological tissues. It can be seen in Fig. 1A that HPLC of an untreated crude extract results in a chromatogram with a number of peaks which would interfere with rapid analysis. However, if extract is first passed through a mini-column of Sephadex G-25, the resulting chromatogram (Fig. 1B) contains only a single peak at the solvent front (2.0 min). Centrifugal elution of these columns is rapid and allows for quantitative separation of macromolecules from low molecular weight substances without dilution[29].

The use of 0.2 *M* SDS in water as an isocratic mobile phase for elution in the HPLC of mono- and polyglutamates of the standard folate analogues results in a rapid separation as seen in Table I. The elution order is consistent with those previously reported for folate polyglutamates on reversed-phase systems[21,22,26] with retention times decreasing as the number of glutamates increases.

Diglutamates are the substrates of choice for the quantitation of hydrolase activity since, unlike longer chain length cogeners, this substrate has only a single γ-glutamyl peptide bond. Thus, the product of hydrolysis cannot subsequently become a substrate. Also, the analysis is not complicated by the endopeptidase activity associated with some hydrolases. Fig. 2A shows the complete separation of IAHQ-Glu from the peak at the solvent front. Progressive hydrolysis of the substrate is shown in Figs. 2B–D. The baseline resolution of the substrate from the product, IAHQ, permits quantitative assessment of hydrolysis rate.

It can be seen in Fig. 3 that the assay is linear over a period of at least 2 h. Further, the amount of product formed is directly proportional to the amount of added extract (Fig. 4). Thus, this method is suitable for the determination of folyl-
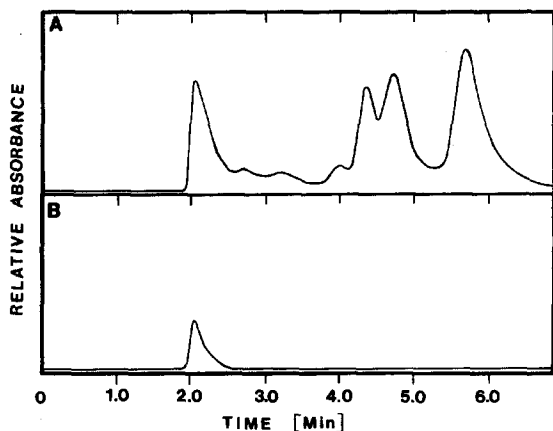

Fig. 1. Chromatogram of mouse kidney extract before (A) and after (B) filtration through Sephadex G-25. Mouse kidney was homogenized in 9 volumes of 0.05 *M* acetate buffer (pH 4.4), autolyzed at 37°C for 60 min and centrifuged at 32 500 *g* for 20 min. An aliquot was passed through a mini-column of Sephadex G-25. Filtered and unfiltered samples were mixed with 0.25 volume of 1 *M* SDS and injected directly onto a Whatman PXS 10/25 ODS column and eluted isocratically with 0.2 *M* SDS.

TABLE I

RETENTION TIMES FOR STANDARD FOLATE ANALOGUES

Separation of standard folate analogues was achieved by isocratic elution on a Whatman PXS 10/25 ODS column with 0.2 M SDS in water at a flow-rate of 1.0 ml/min.

Folate analogue	Retention time (min)
IAHQ	3.92
IAHQ-Glu	2.96
IAHQ-Glu$_2$	2.67
MTX	4.49
MTX-Glu*	3.10
MTX-Glu$_2$	2.59

    * Standard MTX-Glu was not available and the reported retention time is for the product of enzymatic hydrolysis of MTX-Glu$_2$.
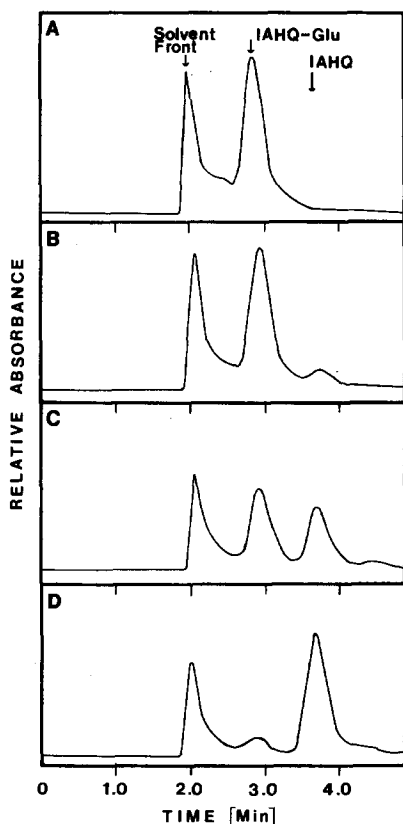

Fig. 2. Hydrolysis of IAHQ-Glu by crude extracts from mouse kidney. Mouse kidney extracts were prepared and filtered through Sephadex G-25 as described in Fig. 1. A 190-μl aliquot of extract was incubated with 85 μm IAHQ-Glu in a total volume of 200 μl for 0 min (A), 15 min (B), 60 min (C), and 120 min (D) at 37°C. Reactions were stopped by introduction of 50 μl of 1 M SDS, 50 μl of each solution was chromatographed as described in Fig. 1.
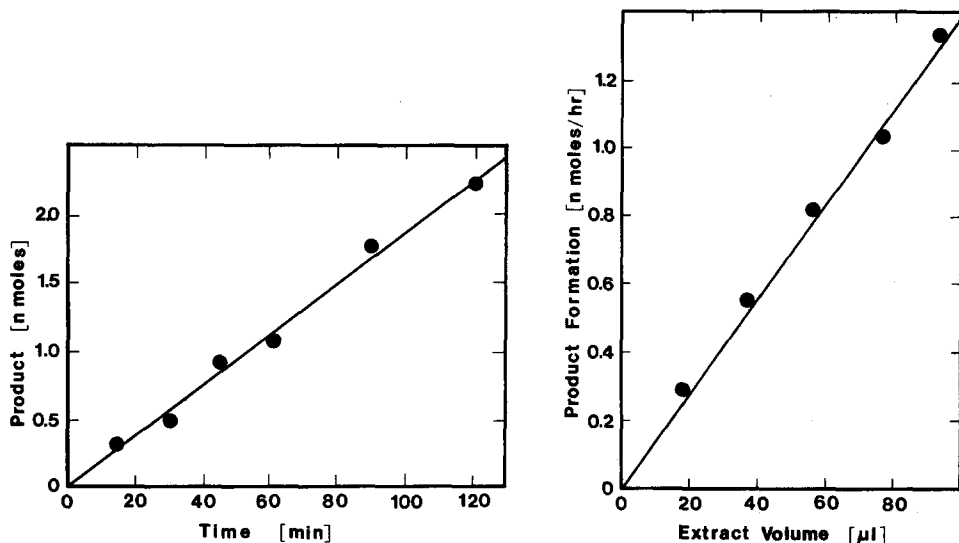
Fig. 3. Time dependence of IAHQ-Glu formation from IAHQ-Glu in the presence of mouse kidney extracts. Extracts were prepared and incubated with IAHQ-Glu at 37°C as described in Fig. 2.

Fig. 4. Hydrolysis rate of IAHQ-Glu as a function of mouse kidney extract volume. Reaction conditions were the same as described in Fig. 2 except that the amount of extract in the reaction mixture (200 μl) was varied as shown.

polyglutamate hydrolase activity in cell-free extracts from mouse kidney. We have applied the assay to other mouse tissues as well (data not shown) with equally successful results and this procedure should prove useful with extracts from a wide variety of sources.

Triglutamates of IAHQ (IAHQ-Glu$_2$) and MTX (MTX-Glu$_2$) can also be separated from the peak at the solvent front and their hydrolysis product peaks in this system (Table I). This could allow investigations of hydrolysis mechanisms, such as endopeptidase *versus* exopeptidase activity and comparison of kinetic constants.

The micellar solvent system used in this assay has several advantages in addition to rapid separation. SDS is an effective protein-denaturing agent and as such can be used to stop reactions without the need for protein precipitation with trichloroacetic acid or heating. Since SDS also has unique solubilizing power, it can be used for direct injection of concentrated protein solutions into the HPLC[30] without time-consuming steps to remove protein precipitates or extraction of folate analogues. SDS is inexpensive and no special organic solvent or other reagents are required. In addition, no time is required for column reequilibration between sample runs because a gradient is not required for complete separation of substrates and products. Therefore, the assay is simple, rapid, inexpensive and applicable to crude hydrolase preparations.

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