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Assay of cytosine and cytidine deaminases by means of reversed-phase high-performance liquid chromatography

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ABSTRACT

A rapid and sensitive high-performance liquid chromatographic assay for cytosine and cytidine deaminases, based upon the chromatographic separation and subsequent ultraviolet detection of enzymatically liberated uracil and uridine, was developed. Using cell-free extract from *Escherichia coli* the enzymes can be assayed with incubation times of 30 min or less. Reversed-phase separation of products from substrates was accomplished by isocratic elution with monobasic ammonium phosphate buffer at pH 3.5. The assay is fast and reproducible with little or no interference from competing reactions in cell extracts. It is sensitive and can concomitantly detect nanomole changes in the concentration of substrate and product. It is faster, more sensitive, and requires fewer sample manipulations than standard spectrophotometric and radiometric methods of analysis.

INTRODUCTION

Cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1) and cytidine deaminase (cytidine aminohydrolase, EC 3.5.4.5) catalyze the hydrolytic deamination of cytosine to uracil and cytidine to uridine, respectively. Both enzymes have been detected in a variety of organisms[1–4] and both play anabolic salvage roles in pyrimidine metabolism in that they supply exogenous pyrimidines *in lieu* of the *de novo* pyrimidine pathway. Both cytosine and cytidine deaminase activities can be determined by direct spectrophotometric assay from fall in absorbance at 285 nm, following conversion of the 4-amino to the 4-keto compounds[5]. When high levels of extraneous protein or certain nucleoside inhibitors are present, the background absorbance becomes too high at 285 nm, the reaction may then be followed at 295 nm. Cytidine deaminase can also be assayed by measuring the amount of labeled uridine formed by the deamination of 2-^{14}C-labeled cytidine[6].

Our laboratories have been examining cytosine deaminases from several bacterial genera including *Pseudomonas*, *Salmonella* and *Escherichia* for some time[1]. The assay for ring deamination at the 4 position based on the spectrophotometric detection of the products and the substrates has some limitations. Cytosine has an

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absorbance maximum at 267 nm while uracil has an absorbance maximum at 259 nm. However, both compounds absorb strongly over a broad range (200–290 nm). Previous assays[3,4] were based on the ability to separate the product from the substrate based on differences on their molar absorptivity at a particular wavelength. In this paper we describe an assay wherein the product and substrate of the reactions are physically separated by a reversed-phase C_{18} high-performance liquid chromatography (HPLC) method prior to measuring the absorbance. Since product and substrate absorb so strongly and since we are not measuring the difference in absorption but the total absorption, the assay is very sensitive and capable of measuring disappearance of substrate and appearance of product in the nanomolar range.

HPLC has been used to monitor enzyme reactions rapidly in a variety of systems[7–9]. There are various advantages for such assays[8]. The assay time is faster than the time course of the reaction, allowing for "real time" assaying of reaction mixture. HPLC methods allow the quantitation of both substrate disappearance (cytidine or cytosine) and product appearance (uridine or uracil) without interference from competing reactions. There is no need to terminate the assay or remove proteins from the assay mix because injection onto the HPLC column effectively terminates the reaction as well as removes and separates protein from the substrates. The enzymatic assay that we describe here overcomes limitations of existing methods while embodying all the above advantages.

EXPERIMENTAL

Chemicals and reagents

Cytidine, cytosine, uridine and uracil were obtained from Sigma (St. Louis, MO, U.S.A.). Monobasic ammonium phosphate was obtained from Mallinckrodt (Paris, MO, U.S.A.). All other chemicals were of analytical grade. Solutions were prepared with distilled deionized water obtained from a Milli-Q system (Millipore, Bedford, MA, U.S.A.).

Growth of cells

Escherichia coli TB2 (*pyrBI, argF, argI*) was grown in M9 minimal medium with 0.2% (w/v) glucose as carbon source[10]. The medium was supplemented with 0.4% casamino acids and uracil at 50 μg/ml. The turbidity was measured with a Klett-Summerson photoelectric colorimeter, using a green filter No. 54. Growth was measured at 37°C and recorded as Klett Units (KU), where 1 KU equals 10^7 cells/ml. Cultures of 1 l at a cell density of 100 KU were harvested and centrifuged at 8000 *g* for 2 min at 4°C. The supernatant was decanted and the cell pellet was used for enzyme extraction. The pellet was suspended in 10 m*M* Tris–HCl (pH 7.0) and broken by explosive decompression using a chilled French Pressure Cell (SLM/AMINCO, Urbana, IL, U.S.A.). The homogenate was centrifuged at 10 000 *g* at 4°C. The pellet was discarded and the supernatant was used for enzyme assay without any further purification. Protein content was determined by the method of Bradford[11], using crystalline bovine serum albumin, Fraction V, as standard.

Cytosine and cytidine deaminase assays

Assays were performed in 1.5-ml microcentrifuge tubes at 37°C in a shaking water bath. The assay mix contained in 1 ml: 10 m*M* Tris–HCl (pH 7.30), 20 μl of appropriately diluted cell extract, and varying concentrations of cytosine or cytidine, as given in the Results and Discussion section. The reaction was terminated after 15–60 min by filtering the entire assay mixture through a 0.45-μm ACRO LC13 filter (Gelman, Ann Arbor, MI, U.S.A.) into a tube stored on ice. A 10-μl volume of this reaction sample was injected onto the column for detection of substrates and products by HPLC. The entire procedure from filtration to injection took less than 30 s. Non-enzymatic oxidative deamination of cytosine or cytidine for the time periods and conditions of temperature and pH employed could not be detected by the subsequent methods of separation and detection described below.

Chromatographic apparatus and conditions

The concentrations of cytidine, cytosine, uridine and uracil were determined using reversed-phase HPLC. The chromatographic system (Waters Assoc., Milford, MA, U.S.A.) consisted of a Model 510 pump, a U6K injector and a variable-wavelength Model 481 LC spectrophotometer. Peaks were integrated manually by paper weighing copies of the output from a Cole Palmer (Chicago, IL, U.S.A.) strip chart recorder or more typically on a Waters Model 740 data module. Samples of 10 μl were injected onto an IBM C_{18} column (250 mm × 4.5 mm I.D.; particle size 5 μm; now supplied by I.I.I Supplies Co., Wallington, CT, U.S.A.). Compounds were separated using isocratic elution (5 m*M* $NH_4H_2PO_4$, pH 3.5) at a flow-rate of 1 ml/min. Compounds were detected by monitoring the column effluent at 254 nm with a sensitivity fixed at 0.1 absorbance units full scale (a.u.f.s.). Individual components of the reaction mixture were identified, using retention times relative to known standards and by injecting known internal standards.

Calculation of K_M values

Initial velocities (V) were measured as a function of substrate concentration (S). Michaelis constants (K_M) for cytosine and cytidine were evaluated from double reciprocal Lineweaver–Burk plots[12]. Uracil formation by cytosine deaminase or uridine formation by cytidine deaminase was linear over the course of the reaction.

RESULTS AND DISCUSSION

Several HPLC procedures have been described for separation of nucleic acid bases and nucleosides. Originally, ion-exchange HPLC[13–16] was used, but subsequently reversed-phase HPLC[17–24] has proven to be more suitable. The reversed-phase techniques included paired-ion chromatography[17], gradient elution[18–22], and isocratic conditions[23,24]. The effects of pH, ionic strength and type of buffer on the reversed-phase separation of nucleosides and bases have been described previously[25]. We have chosen isocratic elution at pH 3.5 to resolve cytosine, uracil, cytidine and uridine.

Typical chromatograms of aqueous standard solutions containing either cytosine and uracil or cytidine and uridine are shown in Fig. 1a and c, respectively. The chromatogram in Fig. 1b gives the results shown after incubation of cytosine with fresh

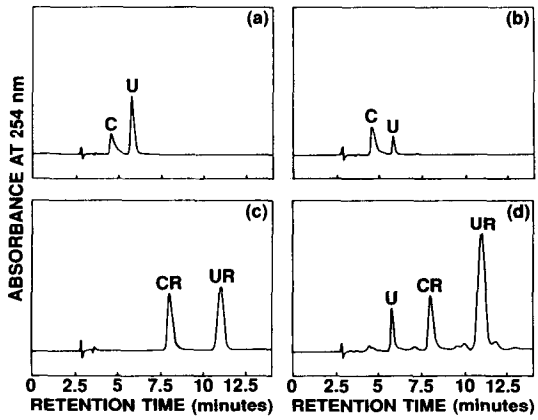

Fig. 1. Chromatograms of aqueous standard solutions of 0.5 m*M* cytosine and uracil (a) and of 0.5 m*M* cytosine after incubation with an enzyme preparation (b). Peaks identified as described in Experimental as cytosine and uracil are labeled C and U respectively. (c) Chromatogram of an aqueous standard solution, containing 1.0 m*M* cytidine and uridine. (d) The enzymatic conversion of cytidine (CR) to uridine (UR) and uracil (U). Graphic reproduction of the chromatogram in Panel D resulted in a change in scale from 0.1 to 0.02 a.u.f.s. The assay mixture contained 1 m*M* cytidine and was treatad as described.

cell extract. The conversion of cytosine to uracil by cytosine deaminase is readily apparent by the concomitant appearance of the uracil peak. The uracil peak was confirmed as pure by both retention time (5.92 *versus* 4.69 for cytosine), as well as inclusion of an internal uracil standard with cytosine. The conversion of cytidine to uridine by cytidine deaminase is shown in Fig. 1d. Incubation of cytidine with cell extract resulted in the appearance of uridine with a concomitant decrease in the cytidine concentration together with the appearance of a uracil peak. Many different organisms have the ability to further degrade uridine to uracil and this is seen in the Fig. 1d. In such cases enzyme activity can be determined by summing the two products or more conveniently by the rate of disappearance of the substrate cytidine. Confirmation of peak purity was made as before with retention times of 8.06 min for cytidine and 11.05 min for uridine. The chromatograms had no extraneous peaks.

The assay was highly reproducible. Assays of the enzymes from different cultures where the cells were grown, harvested, broken and assayed under identical conditions yielded coefficients of variation of less than 12% for cytosine deaminase and less than 11% for cytidine deaminase. Moreover, repeated assays (at least three times) on a single sample gave coefficients of variation of 2.0% or less for both cytosine deaminase and cytidine deaminase.

The reproducibility of the enzymatic reaction was investigated three times for the same cell extract and for different cell extracts of the same strain at 0.5 nmol (cytosine) and 1 nmol (cytidine) and 0.2 mg/ml protein concentrations. The coefficients of variation were 2 and 12% for cytosine deaminase and 2 and 11% for cytidine deaminase.

Thus the assay above, used to detect the presence of the enzymes in crude cell extracts is applicable also for kinetic characterization of cytosine and cytidine deaminases. At a relatively high fixed substrate concentration, the initial velocity

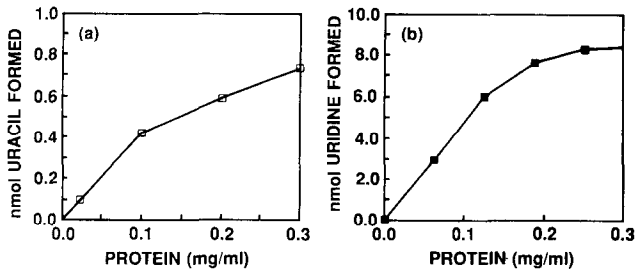

Fig. 2. Effect of enzyme concentration of the rate of the reaction. Cytosine deaminase initial velocity (a) plotted as a function of increasing enzyme concentration; rate of uridine production (b) at increasing enzyme concentration. The assays were performed at constant substrate concentrations of 0.5 m*M* cytosine and 1.0 m*M* cytidine for 30 min, as described in Experimental.

measured was proportional to the amount of enzyme or cell extract employed (Fig. 2). The optimal incubation time for initial velocity measurements was determined by a kinetic assay using a fixed enzyme concentration found to be on the linear portion of the curves shown in Fig. 3a and b. In the example shown, the rate of enzymatic conversion of substrates to products is linear for up to 60 min. Subsequent measurements for kinetic characterization of the enzymes employed sampling times of only 10 min. The incubation conditions, described in Experimental were empirically determined from the above data and represent appropriate linear responses for the variables of enzyme concentration and time of assay. Use of highly purified enzyme or cell extracts with significantly different specific activities may require modifications which are readily determined by additional "range finding" experiments.

To demonstrate the general validity of this assay, we used it to compare the results of our previous determinations of K_M and V_{max} (maximal velocity) for cytosine deaminase[1] that used conventional spectrophotometric assays. The effect of various concentrations of substrate (cytosine or cytidine) on the production of uracil or uridine by crude cell extracts was determined. The enzymes exhibited typical hyperbolic Michaelis–Menten kinetics allowing measurement of the K_M and V_{max} of the two enzymes. Lineweaver–Burk reciprocal plots (Fig. 4) of the experimental data were constructed to measure the effect of increasing substrate concentration on the initial veloc-

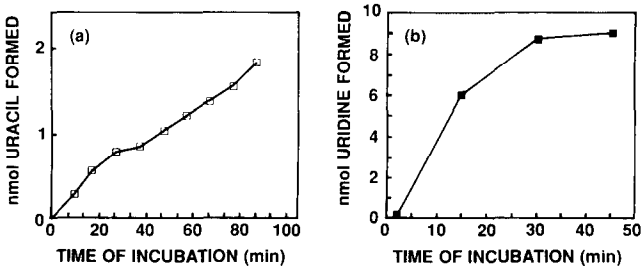

Fig. 3. Time course of the reaction for the production of uracil and uridine by a cell-free extract of *E. coli*. Cytosine deaminase activity (a) was determined at a substrate concentration of 0.5 m*M*. Cytidine deaminase (b) was assayed at a cytidine concentration of 1 m*M*. In every case, 20 μl of cell extract (4 μg protein) was used as described in Experimental.

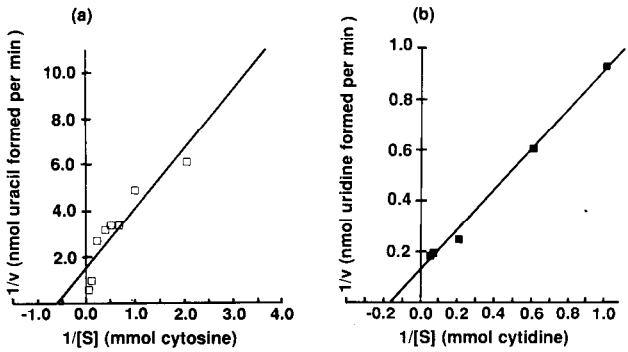

Fig. 4. Lineweaver–Burk plots for cytosine deaminase activity from *E. coli* (a) determined in unpurified preparations of the enzyme. Double reciprocal plot of the production of uridine by cytidine deaminase (b), determined in crude preparations of the enzyme from *E. coli*.

ity. Cytosine deaminase had a K_M of 1.6 m*M* cytosine using data derived from HPLC determinations of enzymatic activity (Fig. 4a) in comparison to a K_M of 1.5 m*M* cytosine for the same sample but determined spectrophotometrically as previously described[1]. Similarly, cytidine deaminase had a K_M of 6.6 m*M* cytidine determined using the assay described, but a K_M of 5.8 m*M* cytidine when assayed spectrophotometrically.

The reversed-phase HPLC determination of cytosine and cytidine deaminase activity described here offers several advantages over currently available assays. Sample preparation is minimal involving no precipitation of protein prior to chromatography. The analysis time, after incubation, is short and the separation is free from interfering substances. The enzymatically liberated uracil and uridine are separated from precursor and measured at 254 nm making detection highly sensitive. Though the results shown were obtained using bacterial cell extracts, the method can be applied to other biological samples.

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