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Determination of Serum Nucleotidase with Cytidine Monophosphate as Substrate

Part II: Improvement of the procedure

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Summary: Serum nucleotidase activity (EC 3.1.3.5) is measured by the amount of ammonia liberated from cytidine after incubation with cytidine deaminase (EC 3.5.4.5). Purification of the cytidine deaminase results in the abolition of interfering reactions, so that routine application is possible.

Bestimmung von Nucleotidase im Serum mit Cytidinmonophosphat als Substrat. Teil II: Verbesserung der Methode

Zusammenfassung: Die Aktivität des Serums gegenüber Cytidin-5′-monophosphat wird bestimmt, indem das durch Inkubation mit Cytidindesaminase freigesetzte Ammoniak gemessen wird. Die Reinigung des Hilfsenzymes beseitigt Störreaktionen und ermöglicht dadurch die routinemäßige Anwendung dieses Prinzipes.

Introduction

Recently, we described a new coupled colorimetric assay for serum activity toward cytidine-5′-monophosphate (5′-nucleotidase activity, EC 3.1.3.5) based on measurement of the equivalent amount of ammonia released from cytidine by cytidine deaminase (EC 3.5.4.5) (1). In developing this assay we used a crude cytidine-deaminase preparation as the auxiliary enzyme, which resulted in rather high background extinction values and an overcorrection with respect to the reagent-control, probably due to an enzyme contaminating the cytidine deaminase. To circumvent this problem, we offered an indirect method. In this communication we prove that a simple and rapid purification of the cytidine deaminase is sufficient to give a reliable direct method, with none of the above disadvantages.

Materials and Methods

Reagents

1. Buffer solution:
   Dissolve 4.20 g sodium diethylbarbiturate and 6.30 g $MgSO_4 \cdot 7H_2O$ in about 800 ml of distilled water. Adjust the pH to 7.50 with HCl (1 mol/l) and dilute to 1,000 ml.
2. Cytidine deaminase solution:
   Dilute the stock cytidine deaminase (see below) with buffer solution to give an activity of about 225 U/l and dissolve 28 mg disodium phenylorthophosphate (British Drug Houses) per 10 ml of this solution (phenylphosphate concentration about 11 mmol/l). Remains stable for about one day at 4°C.
3. Cytidine-5′-monophosphate solution (38.5 mmol/l):
   Dissolve 458 mg cytidine-5′-monophosphate (Boehringer; disodium salt, 6 H_2O, free of cytidine) in 25 ml buffer solution. Prepare just before use.
4. EDTA solution:
   Dissolve 5.6 g EDTA (dipotassium salt, 2 H_2O) in distilled water and make up to 50 ml.
5. Concentrated phenol reagent:
   Dissolve 50 g phenol (A.R. grade) and 0.25 g disodium pentacyano-nitrosylferrate (A.R. grade) in distilled water and make up to 1,000 ml. Stable in an amber bottle at 4°C for at least two months.
6. Concentrated alkali-hypochlorite reagent:
   Dissolve 25 g sodium hydroxide in 60 ml distilled water. Add 72 ml 0.5 mol/l NaClO solution (e.g. from British Drug Houses, in 1 mol/l NaOH) and make up to 1,000 ml. Stable in an amber bottle at 4°C for at least two months.
7. Working phenol/EDTA colour reagent:
   Dilute 1 volume concentrated phenol reagent with 4 volumes distilled water. To 100 ml solution add 2 ml EDTA solution. Prepare fresh before use.
8. Working alkali-hypochlorite colour reagent:
   Dilute 1 volume concentrated alkali-hypochlorite reagent with 4 volumes distilled water. Prepare fresh before use.
9. Cytidine standard solution (3.74 mmol/l):
   Dissolve 92 mg cytidine (Boehringer, more than 99% pure) in saturated benzoic acid and dilute to 100 ml. Stable for months at 4°C.

10. Ammonium sulphate standard solution (1.5 mmol/l buffer solution):
    Dilute 1 volume of a concentrated ammonium sulphate solution (15 mmol/l) with 9 volumes of buffer solution
11. Cytidine solution (50 mmol/l):
    Dissolve 1.22 g cytidine in 100 ml buffer solution. Prepare fresh before use.

Procedures

The 5'-nucleotidase method with cytidine monophosphate

To a series of test tubes, add the amounts indicated in table 1. Before addition of the CMP, the tubes are placed in a 37°C water-bath for a few minutes. After 30 min the reaction is stopped with the phenol colour reagent. After immediate addition and mixing of the alkali-hypochlorite colour reagent, the tubes are incubated for another 30 min at 37°C.

The absorbances of the solutions can then be read at 625 nm

The unknown (A_x) is read against the unknown blank, the standard (A_s) and the reagent control (A_c) against the standard blank. All readings were made in a Zeiss PM Q II spectrophotometer with a 10 mm flow-through-cell.

In the calculations we use the equation:

$$\frac{A_x - A_c}{A_s} \times 124.7 = \text{U/l}$$

The factor $124.7 = \frac{3.74}{10} \times \frac{10.000}{30}$, in which

3.74 = concentration of standard in mmol/l

$\frac{1}{10}$ = volume standard solution (0.1 ml)

10,000 = factor to convert sample volume of 0.1 ml to 1 litre serum

$\frac{1}{30}$ = factor to express the activity per min.

Determination of 5'-nucleotidase activity with adenosine-5'-monophosphate

This is done as described elsewhere (2).

Determination of the cytidine deaminase activity

Dilute the enzyme solution to give a cytidine deaminase activity of about 100 U/l. To a series of tests tubes, add the amounts indicated in table 2.

The unknown (A_x) is read against the unknown blank, the standard (A_s) against the standard blank. In the calculation we use the equation:

$$\frac{A_x}{A_s} \times 100\text{x dilution factor} = \text{U/l}$$

The factor $100 = \frac{1.5 \times 2}{10} \times \frac{10.000}{30}$ is derived from:

1.5 = concentration ammonium sulphate standard (mmol/l).

2 = factor to convert this to NH_3.

$\frac{1}{10}$ = volume standard solution (0.1 ml).

10,000 = factor to convert sample volume of 0.1 ml to 1 litre.

$\frac{1}{30}$ = factor to express the activity per min.

Tab. 1. Procedure for the 5'-nucleotidase determination with cytidine-5'-monophosphate.

	Unknown	Unknown Blank	Reagent Control	Standard	Standard Blank
Serum	0.1 ml	0.1 ml	–	–	–
Cytidine standard solution (9)	–	–	–	0.1	–
Buffer solution (1)	–	0.2 ml	0.1 ml	0.2 ml	0.3 ml
Cytidine deaminase solution (2)	0.8 ml	0.8 ml	0.8 ml	0.8 ml	0.8 ml
CMP solution (3)	0.2 ml	–	0.2 ml	–	–
	Mix thoroughly, stopper, and incubate for 30 min at 37°C. Then rapidly add:				
Phenol/EDTA colour reagent (7)	5 ml	5 ml	5 ml	5 ml	5 ml
Alkali-hypochlorite reagent (8)	5 ml	5 ml	5 ml	5 ml	5 ml
	Mix and incubate for another 30 min at 37°C.				

Tab. 2. Procedure for determination of the cytidine deaminase activity.

	Unknown	Unknown Blank	Standard	Standard Blank
Buffer solution (1)	–	1.0 ml	1.0 ml	1.1 ml
Ammonium sulphate standard solution (10)	–	–	0.1 ml	–
Cytidine solution (11)	1.0 ml	–	–	–
Diluted sample	0.1 ml	0.1 ml	–	–
	Mix and incubate for 30 min at 37°C. Then rapidly add:			
Phenol/EDTA reagent (7)	5 ml	5 ml	5 ml	5 ml
Alkali-hypochlorite reagent (8)	5 ml	5 ml	5 ml	5 ml
	Mix and incubate for another 30 min at 37°C.			

Cytidine deaminase purification method

For maximal yield, *E. coli B* cells (wild type) were grown overnight in nutrient-broth medium (5 g NaCl, 8 g Nutrient-Broth (Difco) and 5 g yeast extract (Difco) per litre distilled water) at 37°C. After reaching the stationary phase, the cells were harvested by centrifugation (15 min 6.000 rpm), and the pellet was taken up in phosphate buffer (10 mmol/l, pH 7.0, containing 1 mmol/l EDTA, according to *Cohen* (3); 8 ml buffer per litre culture fluid). The suspension was then subjected to an ultrasonic treatment for 5 min with cooling (0°C, in MSE Sonic Oscillator). After centrifugation for 10 min at 10,000 *g* the supernatant fluid was subjected to DEAE-cellulose ion-exchange chromatography (Whatman DE 52 in 10 mmol/l phosphate buffer pH 7.0 with 1 mmol/l EDTA; wet settled volume of the ion-exchanger 5 ml per litre culture fluid). The column was washed with 10 mmol/l phosphate buffer (pH 7.0; containing 1 mmol/l EDTA; 40 ml buffer per litre culture fluid) and the cytidine deaminase eluted in 15.5 ml fractons with phosphate buffer (pH 7.0; containing 1 mmol/l EDTA; 25 ml buffer per litre culture fluid; the phosphate content followed a linear gradient from 0.1–0.2 mol/l).

The fractions with peak cytidine deaminase activity were combined and further purified by ammonium sulphate precipitation. The fractions from 40–60% saturation contained the bulk of activity. This precipitate was dissolved in 5 ml phosphate buffer (pH 7.0:10 mmol/l with 1 mmol/l EDTA) and carefully placed on a polyacrylamide-agarose gel column 2.5 cm ϕ X 56 cm; Ultrogel AcA44 – LKB) in phosphate buffer (pH 7.0; 50 mmol/l with 1 mmol/l EDTA). Elution was then performed with the same buffer in 5–15 ml fractions, after which the active fractions were pooled and centrifuged for 2 hours at 10,000 g (4°C). The supernatant fluid is the cytidine deaminase stock solution and was stored at –20°C.

Results

The results of the purification procedure are shown in table 3 for a typical experiment with 12 litres of *E. coli B* culture fluid. Protein was determined according to *Lowry* (4). As can be seen from table 3, the first column (DE-52) removes about 50% of the activity towards CMP. This step is meant only as a preliminary purification to remove the bulk of non-cytidine deaminase proteins and nucleic acids. Good separation between the cytidine deaminase and the activity toward CMP was achieved, especially with the Ultrogel column (fig. 1).

A number of experiments were performed with the purified cytidine deaminase. The 5′-nucleotidase activity of 61 sera was measured, using CMP as substrate. The cytidine deaminase solution was diluted to give about 0.18 U cytidine deaminase per test tube. The same sera were also determined with the AMP method. Figure 2 shows the correlation between these two methods.

Discussion

Several authors have reported lower (3, 5), others higher (6,7) recoveries of isolated cytidine deaminase from various sources. However, their procedures were more complicated than the present method and there is no proof that their preparations fulfil the criteria applied for an auxiliary enzyme in our coupled assay for 5′-nucleotidase activity, i. e. a sufficiently low content of protein to avoid interference in the *Berthelot* reaction and absence of any activity toward CMP (8). The present method is based on these criteria. The content of protein can even be lowered by following the procedure according to *Trimble & Maley* (7), i. e. acidification of the cytidine deaminase solution and subsequent dialysis after centrifugation of the contaminating proteins. On the basis of the negligible interception on the abscissa, the low background absorbance and the good correlation with the AMP method, we conclude that the proposed method makes it possible to estimate serum 5′-nucleotidase with improved sensitivity.

Tab. 3. Results of purification method

	Volume (ml)	Protein		absorbance at		activity toward cytidine			activity toward CMP*)			cytidine deaminase/ CMP deaminase
		(g/l)	total (g)	260 nm	280 nm	(I.U./l)	total (I.U.)	(I.U./mg protein)	(I.U./l)	total (I.U.)	(I.U./g protein)	
Crude extract (after ultrasonic treatment and 10,000 *g* centrifugation	90	53.8	4.85	357	205	13,480	1,212	0.25	297	26.7	5.52	45
DE-52 eluate (peak fractions combined)	93	6.48	0.60	10.38	8.66	6,580	612	1.02	37.8	3.51	5.83	174
Ammonium sulphate (active fractions combined, dialysed and concentrated)	5	–	–	–	47.2	64,720	324	–	195.3	0.98	–	331
Ultrogel AcA44 – eluate	20	2.79	0.06	1.81	3.18	10,880	218	3.6	1.5	0.03	0.55	7,260

*) After dialysis to remove NH_3

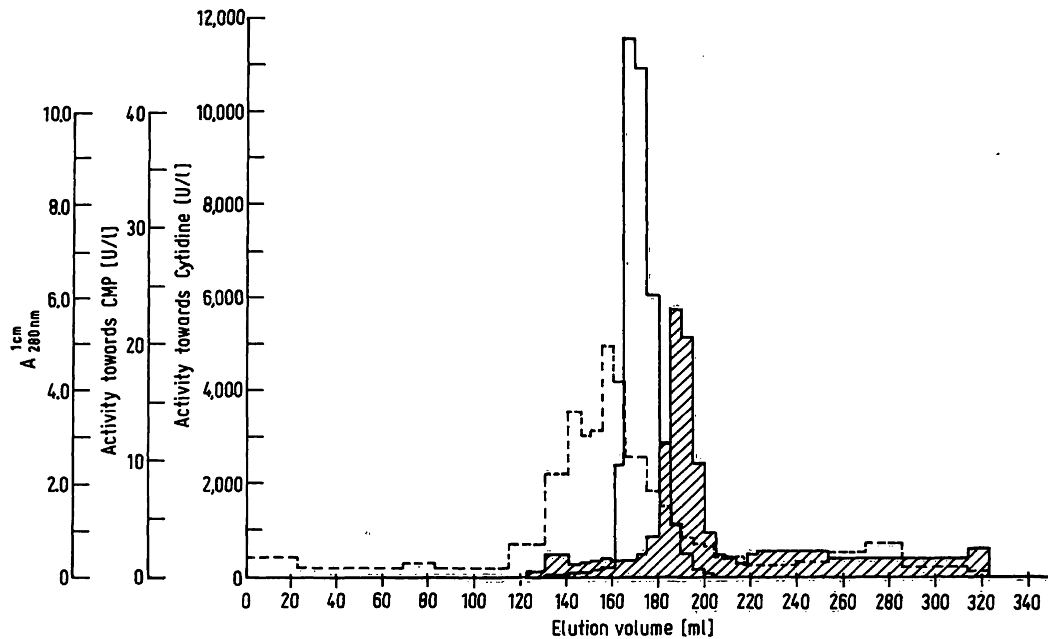

Fig. 1. Elution of protein (absorbance at 280 nm: ---), activity toward cytidine (——) and toward CMP (shaded) from an Ultrogel AcA44 column.

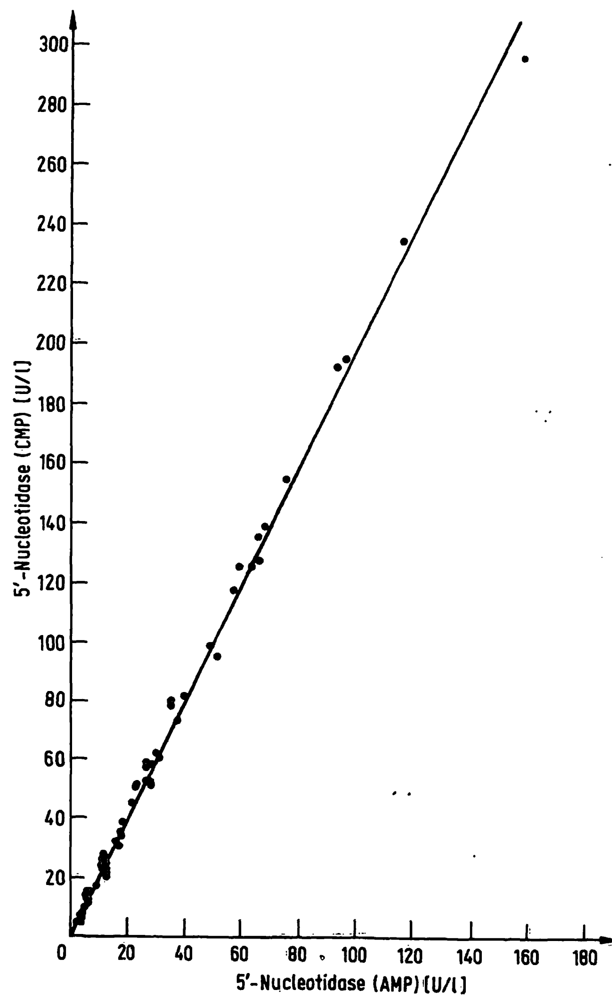

Fig. 2. Correlation between the two 5'-nucleotidase methods (AMP or CMP as substrate) for 61 sera. Calculated regression equation: y (CMP) = 1.98 x (AMP) − 0.11. Correlation coefficient r = 0.998.

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