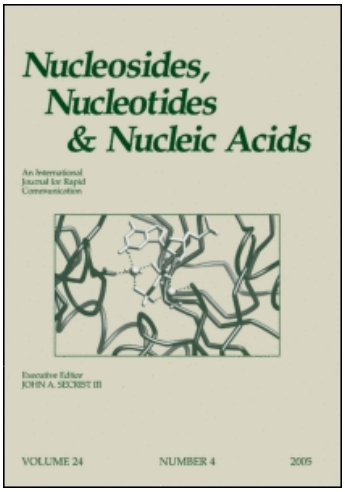

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A NOVEL AFFINITY CHROMATOGRAPHY METHOD FOR THE CO-PURIFICATION OF DEOXYCYTIDINE KINASE AND CYTIDINE DEAMINASE

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A NOVEL AFFINITY CHROMATOGRAPHY METHOD FOR THE CO-PURIFICATION OF DEOXYCYTIDINE KINASE AND CYTIDINE DEAMINASE

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ABSTRACT

By affinity chromatography with Sepharose coupled to 2′-deoxy-1-β-D-ribofuranosyl-N^4-dodecanoylcytosine, deoxycytidine kinase and cytidine deaminase were purified 1,950- and 2,240-fold, respectively, from Ehrlich carcinoma cells, and their enzyme activities for several deoxycytidine analogs were investigated.

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INTRODUCTION

Deoxycytidine kinase (dCK, EC 2.7.1.74) catalyzes the phosphorylation of 2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine, and a variety of their nucleoside analogs in the presence of nucleoside triphosphates as phosphate donors[1]. Its activity is essential for the activation of a number of chemotherapeutically important nucleoside analogs[2–4]. Cytidine deaminase (CDA, EC 3.5.4.5) catalyzes the deamination of cytidine and 2′-deoxycytidine. A number of chemotherapeutically important 2′-deoxycytidine analogs including 1-β-D-arabinofuranosylcytosine (AraC) and 2′-*C*-cyano-2′-deoxy-1-β-D-*arabino*-pentofuranosylcytosine (CNDAC) are metabolized to inactive uracil derivatives by CDA, although they are poorer substrates than cytidine and 2′-deoxycytidine[5–7].

In our previous report, we have shown that Sepharose 4B coupled to 2′-*C*-cyano-2′-deoxy-β-D-*arabino*-pentofuranosyl-N^4-palmitoylcytosine (p-CNDAC), a lipophilic derivative of CNDAC, is a useful affinity gel for the purification of dCK from mouse Ehrlich carcinoma cells[8]. However, Sepharose 4B coupled to CNDAC or 2′-deoxycytidine could not be used for purifying the enzyme. These results suggest that the palmitoyl moiety of p-CNDAC-immobilized Sepharose 4B serves as a long spacer necessary for binding of the enzyme to the ligand.

We examined whether the gel coupled to 2′-deoxycytidine, via a long spacer, is a useful affinity gel for purifying the enzyme. By affinity chromatography using Sepharose 4B coupled to 2′-deoxy-1-β-D-ribofuranosyl-N^4-dodecanoylcytosine (DRDC), CDA as well as dCK could be purified from Ehrlich carcinoma cells. In this paper, the purification of the enzymes from Ehrlich carcinoma cells using DRDC-immobilized Sepharose and their activities for CNDAC, 2′-deoxy-2′-methylidenecytidine (DMDC)[9], and 2′-deoxy-2′-*C*-methylcytidine (SMDC)[10] were described.

MATERIALS AND METHODS

Nucleosides and nucleotides were obtained from Sigma Chemical Co. (St. Louis, MO). CNDAC, DMDC, and SMDC were synthesized as described previously[9–11]. All other chemicals were of analytical grade.

Ehrlich carcinoma cells, recovered directly from the peritoneal cavity, were supplied by the Sloan-Kettering Institute, New York, NY, and have been maintained in our institute in ascites form. The cells obtained were washed twice by centrifugation and kept frozen at $-85\,^{\circ}C$ until use.

2′-Deoxy-1-β-D-ribofuranosyl-N^4-(12-amino)dodecanoylcytosine was prepared from 2′-deoxycytidine and 12-aminododecanoic acid according to the method reported by Kaneko *et al*[12]. To prepare DRDC-immobilized Sepharose, 2′-deoxy-1-β-D-ribofuranosyl-N^4-(12-amino)dodecanoylcytosine

was coupled to ECH-Sepharose 4B (Amersham Pharmacia Bioteh, Buckinghamshire, UK) according to the manufacturer's protocol. Typically, 2–4 μmole of DRDC bound to 1 ml of Sepharose 4B.

Analysis by HPLC and measurement of protein concentration were done as described previously[8]. dCK activity was measured using UTP as a phosphate donor because UTP is a better donor than ATP[8,13]. Twenty μl of 200 mM Tris-HC1 buffer, pH 7.5, containing 1.0 mM 2′-deoxycytidine, 1.0 mM UTP, 4.8 mM $MgCl_2$, 2 mg/ml bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), 2 mM NaF, and 2 mM tetrahydrouridine was added to 20 μl of enzyme solution. The mixture was incubated at 37 °C for 10 min, followed by HPLC analysis at 280 nm. CDA activity was measured by the following method. Twenty μl of 200 mM Tris-HC1 buffer, pH 7.5, containing 1.0 mM 2′-deoxycytidine, 4.8 mM $MgCl_2$, 2 mg/ml BSA, and 1 mM DTT was added to 20 μl of enzyme solution. The mixture was incubated at 37 °C for 10 min, followed by HPLC analysis at 260 nm.

Ehrlich carcinoma ascites cells, 18–20 g wet weight, were suspended in buffer A (10 mM imidazole hydrochloride buffer, pH 6.5, containing 5 mM DTT, 100 μM 1,10-*O*-phenanthroline, and 100 μM phenylmethanesulfonyl fluoride (PMSF)) and homogenized for 5 min with a Polytron homogenizer. The lysed cell extract was centrifuged at $10{,}000 \times g$ for 30 min. The resulting supernatant was fractionated with ammonium sulfate. The protein fraction precipitating at a level between 30% and 60% saturation was dissolved in buffer B (50 mM imidazole hydrochloride buffer, pH 6.5, containing 5 mM DTT, 24 mM $MgCl_2$, 0.2 M KCI, 5% glycerol, 100 μM 1,10-*O*-phenanthroline, and 100 μM PMSF). The fraction was applied to a DRDC-immobilized Sepharose column (vol. 1 ml) equilibrated with buffer B. The column was washed with 10 ml of buffer B containing 1.0 M NaCl and then 10 ml of buffer C (50 mM imidazole hydrochloride buffer, pH 6.5, containing 5 mM DTT, 24 mM $MgCl_2$, and 5% glycerol). The fractions eluted from the column by these treatments had no dCK or CDA activity. The colunm was eluted with 10 ml of Buffer C containing 10 mM 2′-deoxycytidine and 0.5 ml fractions were collected. Fractions showing enzymatic activity were pooled and desalted using a PD-10 column (Amersham Pharmacia Biotech, Buckinghamshire, UK). Glycerol (20% v/v) and 1 mg/ml BSA were added to the desalted fractions, which were subsequently stored at −85 °C.

RESULTS AND DISCUSSION

2′-Deoxycytidine was converted to deoxycytidine 5′-monophosphate by the final enzyme preparation from Ehrlich carcinoma cells in the presence of UTP and tetrahydrouridine, whereas 2′-deoxycytidine was converted to 2′-deoxyuridine by the preparation in the absence of the phosphate donor and

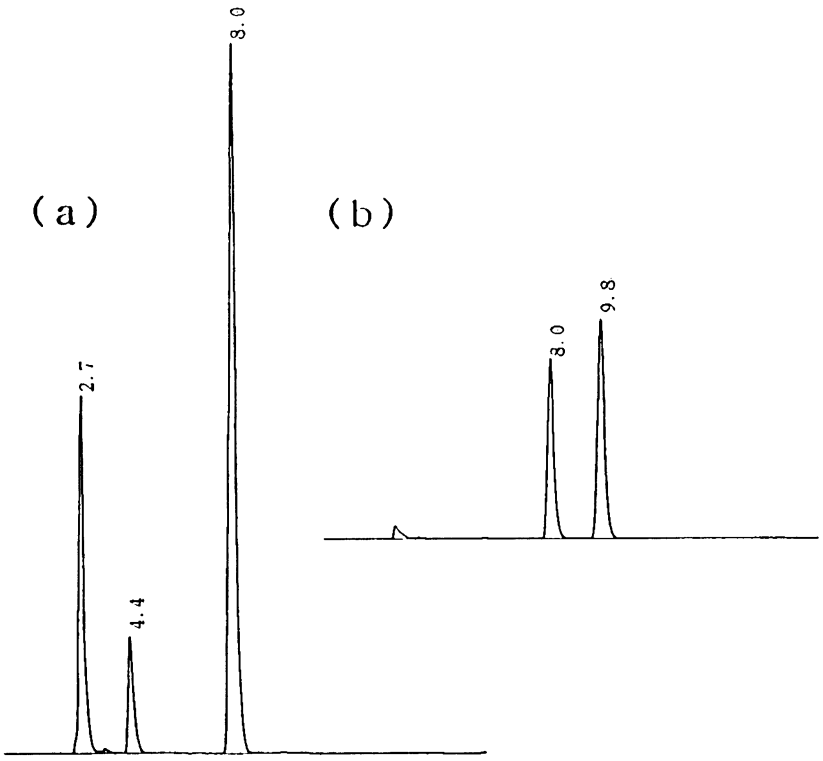

Figure 1. HPLC chromatogram of 2′-deoxycytidine incubated with the enzyme preparation from Ehrlich carcinoma cells. (a) Twenty μl of 200 mM Tris-HCl buffer, pH 7.5, containing 1.0 mM 2′-deoxycytidine, 1.0 mM UTP, 4.8 mM $MgCl_2$, 2 mg/ml BSA, 1 mM DTT, and 2 mM tetrahydrouridine was added to 20 μl of enzyme solution. After the mixture was incubated at 37°C for 10 min and HPLC analysis was done at 280 nm. Retention time: UTP; 2.7 min UDP; 2.7 min, 2′-deoxycytidine 5′-monophosphate; 4.4 min, 2′-deoxycytidine; 8.0 min. (b) Reaction was done at the absence of UTP and tetrahydrouridine and the product was analyzed at 260 nm. Retention time: 2′-deoxycytidine; 8.0 min, 2′-deoxyuridine; 9.8 min.

the cytidine deaminase inhibitor (Fig. 1). These results suggest that the preparation obtained has dCK and CDA activities. The preparation showed neither phosphatase activity for UTP and deoxycytidine 5′-monophosphate nor phosphorylase activity for deoxycytidine 5′-monophosphate in the presence of ATP and UTP (data not shown).

The purification results of dCK and CDA are summarized in Table 1. The activities of dCK and CDA were purified 1,950- and 2,240-fold, respectively, as compared with those of the crude extract. The yields of both enzymes were approximately 50%.

The purification and characterization of dCK and CDA in various tumor cells are important for the examination of tumor cell drug sensitivity[14].

Table 1. Purification of dCK and CDA from Ehrlich Carcinoma Cells. Reaction and HPLC Analysis Were Done as Described in Materials and Methods Except That the Reaction Mixtures Containing Cell Free Extract or $(NH_4)_2SO_4$ Fraction Were Incubated for 120 min

	dCK			CDA		
	Total Activity (nmol/min)	Specific Activity (nmol/mg)	Purification	Total Activity (nmol/min)	Specific Activity (nmol/mg)	Purification
Cell free extract	94.5	0.176	1	310	0.577	1
$(NH_4)_2SO_4$ fractionation	87.5	0.325	1.8	275	1.34	2.3
DRDC-Sepharose	43.8	343	1,950	165	1,290	2,240

The application of suitable affinity chromatography methods to the purification of dCK and CDA is critical as these enzymes are unstable and comprises only a small fraction of total cell proteins. Deoxycytidine 5′-adenosine 5″-p1,p4-tetraphosphate-, dTTP-, dCTP-, and deoxycytidine 5′-tetraphosphate-immobilized Sepharose have been shown to be useful in the purification of dCK from human leukemias and lymphomas[15–18]. We have shown that Sepharose 4B coupled to p-CNDAC is a useful affinity gel for the purification of dCK from mouse Ehrlich carcinoma cells[8]. 6-[3-(5-Cytidyl)acryloylamino]hexanoic acid bound agarose resin is used to purify CDA from *Escherichia coli* and human tissues[19,20]. To our knowledge, this report might be first one that dCK and CDA could be co-purified by affinity chromatography using an affinity gel. The column was used repeatedly over a period of three months. Our preliminary experiment showed that dCK and CDA in the cell free extract from human HT-1080 fibrosarcoma cells bound to DRDC-immobilized Sepharose column and that the enzyme activities were observed in the fractions eluted from the column by the treatment with buffer C containing 10 mM 2′-deoxycytidine. DRDC-immobilized Sepharose might be a useful affinity gel for the purification of dCK and CDA from various tumor cells.

The kinetic parameters of the purified CDA for 2′-deoxycytidine and cytidine are shown in Table 2. The V_{max} and K_m for cytidine were 3.5- and 4.4-fold higher than those for 2′-deoxycytidine, respectively, whereas V_{max}/K_m were similar. The V_{max} and K_m of CDA from human placenta for cytidine were comparable to those for 2′-deoxycytidine[15]. The K_m for the substrates of CDA from Ehrlich carcinoma cells were two-magnitude higher than those of CDA from human placenta. These might reflect differences in species and tissue sources.

Table 2. K_m and V_{max} of CDA for 2′-Deoxycytidine and Cytidine. Reaction Mixture Containing 100 mM Tris-HCl Buffer, pH 7.5, 0.05–1.0 mM Substrate, 2.4 mM $MgCl_2$, 1 mg/ml BSA, 0.5 mM DTT, and Enzyme in a Total Volume of 40 μl Was Incubated at 37 °C for 5 min, Followed by HPLC Analysis at 260 nm

Substrate	K_m (mM)	V_{max} (nmole/min ml)	V_{max}/K_m
2′-Deoxycytidine	0.14	57	410
Cytidine	0.49	250	510

The enzymatic activities of the purified dCK and CDA for 50 μM AraC, CNDAC, DMDC, and SMDC which have antitumor activities are shown in Table 3. The phosphorylation rates of the respective analogs by dCK decreased in the order of AraC > SMDC > DMDC > CNDAC. AraC and CNDAC were deaminated by CDA, but DMDC and SMDC were not. The deamination rate of CNDAC was 6-fold smaller than that of AraC. Co-purification of dCK and CDA by affinity chromatography with DRDC-Sepharose could make it possible to measure the activities of the enzymes from a tumor for cold cytidine analogs.

Knowledge about the substrate properties of cytidine analogs for dCK and CDA from each of various human and mouse tumors contributes to the design of antitumor agents and better application of antitumor cytosine nucleosides for the treatment of cancer. To co-purify dCK and CDA, DRDC-immobilized Sepharose may be better than the other

Table 3. Activities of dCK and CDA from Ehrlich Carcinoma Cells for 50 μM Several 2′-Deoxycytidine Analogs

	dCK		CDA	
	Activity[a] (nmol/min ml)	Relative Activity	Activity[b] (nmol/min ml)	Relative Activity
2′-Deoxycytidine	6.3	100	25	100
AraC	5.3	84	6.1	24
CNDAC	2.6	41	1.0	4.0
DMDC	3.1	49	0	0
DMDC	4.5	71	0	0

a) Reaction mixture containing 100 mM Tris-HCl buffer, pH 7.5, 50 μM each analog, 0.5 mM UTP, 2.4 mM $MgCl_2$, 1 mg/ml BSA, 0.5 mM DTT, 1 mM NaF, 1 mM tetrahydrouridine, and enzyme in a total volume of 40 μl was incubated at 37 °C for 15 min, followed by HPLC analysis at 280 nm. Each phosphorylation was confirmed by conversion of the product to the substrate by phosphatase alkaline, after incubation overnight.
b) Reaction was done as described in the head note to Table 2 except that 50 μM each analog was used as the substrate.

affinity gels including Sepharose 4B coupled to p-CNDAC. The application of DRDC-immobilized Sepharose for the purification of enzymes from various sources and investigation of the activities of these enzymes for various cytidine analogs, with or without anti-tumor activities, are in progress.

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REFERENCES

1. Arnér, E.S.J.; Eriksson, S. Pharmac. Ther. **1995**, *67*, 155–186.
2. Veronique, W.T.; Ruiz van Haperen; Peters, G.J. Pharm. World Sci. **1994**, *16*, 104–112.
3. Obata, T.; Endo, Y.; Tanaka, M.; Matsuda, A.; Sasaki, T. Cancer Lett. **1998**, *123*, 53–61.
4. Stegmann, A.P.A.; Honders, W.H.; Willemze, R.; Ruiz, V.W.T.; Landegent, J.E. Blood **1995**, *5*, 1188–1194.
5. Muller, W.E.G.; Zahn, R.K. Cancer Res. **1979**, *39*, 1102–1107.
6. Tanaka, M.; Matsuda, A.; Terao, T.; Sasaki, T. Cancer Lett. **1992**, *64*, 67–74.
7. Eliopoulos, N.; Cournoyer, D.; Mompartler, R.L. Cancer Chemother. Pharmacol. **1998**, *42*, 373–378.
8. Uchida, H.; Chen, Y.-X.; Morinaga, H.; Hayashi, Y.; Matsuda, A.; Obata, T.; Endo, Y.; Sasaki, T. Biol. Pharm. Bull. **1999**, *22*, 83–86.
9. Matsuda, A.; Takeuchi, K.; Sasaki, T.; Ueda T.J. Med. Chem. **1991**, *31*, 234–239.
10. Matsuda, A.; Takeuchi, K.; Tanaka, M.; Sasaki, T.; Ueda, T.J. Med. Chem. **1991**, *31*, 812–819.
11. Azuma, A.; Nakajima, Y.; Nishizono, N.; Miyakawa, N.; Suzuki, M.; Hanaoka, K.; Kobayashi, T.; Tanaka, M.; Sasaki, T.; Matsuda, A.J. Med. Chem. **1993**, *36*, 4183–4189.
12. Kaneko, M.; Koga, R.; Murayama, K.; Shibata, T.; Hotoda, H.; Suzuki, M.; Hanaoka, K.; Tanzawa, F.; Kurakata, S.; Kobayashi, T.; Sasaki, T.; Matsuda, A. Proc. Amer. Assoc. Cancer Res. **1997**, *38*, 679.
13. White, J.C.; Hines, L.H. Cancer Res. **1987**, *47*, 1820–1824.
14. Colly, L.P.; Peters, W.G.; Richel, D.; Arentsen-Honders, M.W.; Starrenburg, C.W.; Willemze, R. Semin. Oncol. **1987**, *14*, 257–261.
15. Bohman, C.; Eriksson, S. Biochemistry **1988**, *27*, 4258–4265.
16. Datta, N.S.; Schewach, D.S.; Hurley, M.C.; Mitchell, B.S.; Fox, I.H. Biochemistry **1989**, *28*, 114–123.

17. Wang, L.-M.; Kucera, G.L.; Capizzi, R.L. Biochem. Biophys. Acta **1993**, *1202*, 309–316.
18. Kim, M.-Y. J. Biochem. Mol. Biol. **1995**, *28*, 281–289.
19. Ashley, G.W.; Bartlett, P.A. J. Biol. Chem. **1984**, *21*, 13615–13620.
20. Cacciamani, T.; Vita, A.; Cristalli, G.; Vincezetti, S.; Natalimi, P.; Ruggieri, S.; Amici, A.; Magni, G. Arch. Biochem. Biophys. **1994**, *290*, 5401–5407.

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