YALE JOURNAL OF BIOLOGY AND MEDICINE 69 (1996), pp. 495-503.

3′-Amino Thymidine Affinity Matrix for the Purification of Herpes Simplex Virus Thymidine Kinase

Peter P. Tung, John Respass, and William C. Summers[a]

Departments of Therapeutic Radiology, and Molecular Biophysics and Biochemistry, and Genetics, Yale University School of Medicine, New Haven, Connecticut

(Received June 7, 1995; returned for revision August 5, 1995; accepted June 1, 1997)

A simple procedure for preparation of an affinity resin with 3′-amino thymidine linked to the carboxyl residues on 6-amino-hexanoic agarose is described. We have used this column for a rapid and simple purification of the thymidine kinase encoded by the herpes simplex virus type 1 genome. This resin has two major advantages over the most widely use used resin made with thymidine-p-nitrophenyl phosphate: first it is easily obtainable, and second, it is not subject to destruction by phosphodiesterases. The two resins are very similar in behavior and the resin made with amino thymidine has allowed us to prepare large quantities of highly purified HSV TK for crystallization studies.

INTRODUCTION

While affinity chromatography provides a powerful tool for the purification of many proteins, the availability of suitable affinity resins or the ligands needed to prepare such resins may limit the use of this approach to protein purification. In our studies of the thymidine kinases encoded by several of the herpesviruses [1, 2], we have employed the affinity resin described by Kowal and Marcus [3] which is prepared by coupling the affinity ligand thymidyl-3′-p-amino-phenol via the free amine to the carboxyl group on CH-sepharose in a reaction mediated by a water-soluble carbodiimide. This ligand is easily prepared from the nitro derivative by catalytic reduction with hydrogen at one atmosphere pressure. For a number of years this nitro compound, thymidine-p-nitrophenyl phosphate has been commercially available as a chromogenic substrate for certain phosphodiesterases [4]. Recently, however, this starting material has become commercially unavailable. While the synthesis of this compound is relatively straightforward, we have investigated other ligands to see if a simple and reliable method could be devised to make a useful thymidine-containing affinity resin for purification of the herpesvirus thymidine kinases.

We have found that a resin prepared by linking 3′-amino thymidine via the amino group to a 6-carbon linker arm on sepharose works as well as, if not better than, the resin described by Kowal and Marcus. The 3′-amino thymidine resin has two advantages over the formerly used resin; first, it is less expensive to prepare and second, it is not subject to release of the ligand from the column by contaminating phosphodiesterases.

[a] *To whom all correspondence should be addressed:* William C. Summers, M.D., Ph.D., Radiobiology Section, Yale University School of Medicine, 333 Cedar St, New Haven, CT 06520-8040. Tel.: 203-785-2986; Fax: 230-785-6309; E-mail: william.summers@yale.edu.

[b] *Abbreviations:* AMT, 3′-amino thymidine; AZT, 3-azido thymidine; DEAE, diethylaminoethyl; DTT, dithiothreitol; EBV, Epstein-Barr virus; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HPLC, high pressure liquid chromatography; HSV, herpes simplex virus; IPTG, isopropyl thiogalactoside; SDS, sodium dodecyl sulfate; TdR, thymidine; TK, thymidine kinase.

MATERIALS AND METHODS

Reagents

3′-Amino thymidine (AMT), 3′-azido thymidine (AZT), thymidyl-3′-p-nitrophenol (p-nitro-phenyl-phosphate thymidine), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC), and 6-amino-hexanoic agarose (CH-Sepharose 4B)were purchased from Sigma Co. Palladium on carbon catalyst was purchased from Aldrich Chemical Co. and hydrogen gas was obtained from New Haven Compressed Gasses, Inc.

Enzymes

Herpes simplex thymidine kinase (HSV TK) was purified to homogeneity from *E. coli tdk* strain SY211 [5] which contained the pET8c:HSVTK [6, 7] expression plasmid. The purification procedure was described in Sanderson et al. [1]. Thymidine kinase activity was assayed by the standard assay described by Summers and Summers [8].

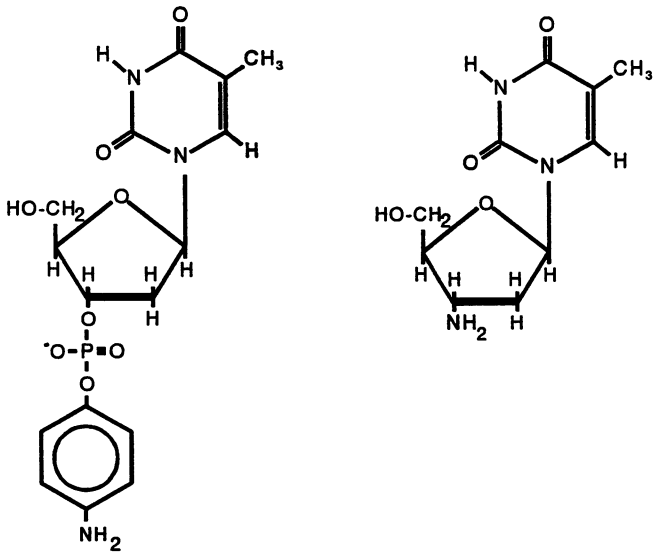

Figure 1. Structures of ligands used. Left: Thymidyl-p-amino phenol. Right: 3′-Amino thymidine.

Synthesis of 3′-amino thymidine

AMT (Figure 1) was synthesized in one step from AZT using a simple modification of the procedure of Lin and Prusoff [9]. One gram of AZT (3.7 mmole) and 0.5 gm of palladium-carbon catalyst was added to 200 ml absolute ethanol in a 250 ml Erlenmeyer flask. Hydrogen gas was slowly bubbled through this solution from a Pasteur pipette for 24 hr in a fume hood. Ethanol was periodically added to maintain the volume at 200 ml. At the end of the reaction, the catalyst was removed by filtration and the filtrate evaporated to dryness by lyophilization. The residue was taken up in 50 ml water, adjusted to pH 3 with HCl, and applied to a 2.5 x 26 cm Dowex AG50W-X8 column which was washed with 1000 ml distilled water and then eluted with 200 ml of 1 M NH_4OH as described by Lin and Prusoff [9]. The product was about 85 percent AMT and about 15 percent AZT as

determined by HPLC analysis on an ion exchange column using the commercial samples from Sigma as standards. For this HPLC analysis, samples were applied to a Ranin hydropore anion exchange column (10 x 100 mm) and eluted over 15 min with a linear gradient starting with 5 mM potassium phosphate, pH 4.0 and ending with 1 M potassium phosphate, pH 4.0. AMT and AZT were detected and quantitated by optical absorbance at 267 nm.

Preparation of AMT-sepharose affinity resin

AMT was coupled to the terminal carboxyl group of CH-Sepharose 4B according to the procedure recommended by the manufacturer, Pharmacia. A calculated equimolar amount of AMT in water was adjusted to pH 5 with HCl and added to washed CH-Sepharose. Dry EDAC was added to give a final concentration of 100 mM and the slurry was gently stirred at room temperature. The pH was adjusted and maintained at 5.0 for the first hour by the addition of HCl. The coupling proceeded for 24 hr at room temperature. To block any unreacted carboxyl groups, 100 mM tris Cl, pH 5, was added along with additional EDAC to raise the total concentration to 150 mM and the reaction allowed to proceed for two more hours. The gel was washed first with 1 M NaCl in 0.1 M NaOAc, pH 4.0, then with 1 M NaCl, 100 mM tris Cl, pH 8.0, and finally with distilled water. The derivatized gel was stored at 4°C in 50 mM tris, pH 7.5, 10 percent glycerol, 0.2 percent NaN_3.

To estimate the efficiency of coupling, for one reaction with pure commercial AMT, the amount of starting material was determined by its optical absorbance of the solution (with the gel removed by centrifugation) prior to the coupling reaction; similarly, the amount of unreacted AMT remaining was estimated at the end of the reaction.

Preparation of the thymidyl-p-amino phenol derived resin was prepared as described by Kowal and Marcus [3] by catalytic reduction of the nitro derivative to give the amino compound (Figure 1) followed by coupling to CH-Sepharose with EDAC.

Affinity chromatography

Affinity chromatography was carried out at room temperature to increase the affinity of the enzyme for the resin [10]. For analytical evaluation and comparisons, 1 ml of each resin was packed in a 3 ml plastic syringe plugged with glass wool. Prior to loading the samples, each column was equilibrated with 20 mM tris Cl, pH 7.5, 10 percent glycerol, 3 mM dithiothreitol (DTT). Samples of purified HSV TK, generously supplied by Celine Melitz of this laboratory, were diluted into 0.5 ml of this equilibration buffer and allowed to run into the columns. The columns were then washed with 3 ml 0.1 M tris Cl, pH 7.5, 10 percent glycerol, 3 mM DTT (Low Salt Wash), followed by 0.3 M tris Cl, pH 7.5, 10 percent glycerol, 3 mM DTT and thymidine at the indicated concentrations (High Salt Wash). For some experiments, a linear gradient of increasing thymidine concentration was used to elute the enzyme; in other experiments one or more steps in thymidine concentration were used.

Fractions (0.5 ml) were collected and assayed for optical absorbance at 260 nm to monitor the thymidine concentration and by enzymatic assay to detect TK activity. Selected fractions were also analyzed by electrophoresis in SDS-containing polyacrylamide gels.

For preparative experiments, larger columns were used (2.5 cm x 7 cm) and an additional wash of High Salt Wash without thymidine was added after the Low Salt Wash to elute more nonspecific, electrostatically adsorbed proteins.

Large-scale purification of HSVTK

A modification of the procedure of Krane [Yale University MS Thesis, 1981] was used to purify HSVTK from bacterial cells containing plasmid expression vectors. Twelve liters of Luria-Bertani broth supplemented with 100 μg/ml ampicillin were inoculated from an overnight culture of *E. coli* SY211 with plasmid pET8c:HSVTK and grown at 37°C with strong aeration. When the cultures were in mid-exponential growth (ca. 3 x 10^8 cells/ml) IPTG was added to 1 mM to induce the expression of the HSVTK from the vector plasmid. After three hr of continued incubation and shaking, the cells were harvested by centrifugation. The cell pellet (22 gm) was resuspended in 100 ml buffer A (50 mM tris Cl, pH 7.5, 0.2 mM TdR, 1 mM DTT, 10 percent glycerol) and frozen (-20°C) and thawed twice. Ten mg of lysozyme and triton X100 (0.1 percent final concentration) were added and the partially lysed cell suspension was then placed in a chilled blender with an equal volume of glass beads (ca 0.05 mm) and the cells broken by blending at high speed for 5 min (in one minute bursts). The temperature was monitored and not allowed to exceed 15°C. The broken cell mass was centrifuged at 8,000 rpm for 10 min in the J17 rotor of a Beckman refrigerated centrifuge (4°C) to remove the glass beads and cellular debris. The beads were washed with 50 ml cold buffer A, recentrifuged, and the supernatants combined to yield crude extract which was adjusted to 150 ml with buffer A. All subsequent steps were carried out at 4-8°C unless noted to the contrary. The crude extract was fractionated by addition of 52.5 gm ammonium sulfate (50 percent saturation). After overnight precipitation at 4°C, the insoluble proteins (which included the HSVTK activity) were collected by centrifugation at 8,000 rpm for 30 min (4°C) and resuspended in 150 ml buffer A. The next step was a batch adsorption with DEAE to remove bulk nucleic acids and some contaminating proteins. The crude extract was mixed with 50 ml washed DEAE-cellulose (Whatman DE-52) and stirred for 30 min. This mixture was loaded into a glass column (4 cm diameter) and the flow-through collected. The DEAE-cellulose was washed with 100 ml buffer A. Again the flow-through was collected and combined with the initial material. The combined DEAE eluate was 210 ml. Prior to affinity chromatography it was necessary to eliminate the thymidine in the buffer, so this material was precipitated with ammonium sulfate (50 percent saturated) and resuspended in 75 ml Low Salt Buffer (see above). This material was then loaded on a 2.5 cm x 7 cm AMT affinity column which was operated at room temperature. The column was washed with 100 ml Low Salt Buffer and then 50 ml High Salt Buffer. The thymidine kinase was eluted with 75 ml of High Salt Buffer containing 0.2 mM thymidine. Fractions (3 ml) were collected and all fractions with thymidine (i.e., as determined by increase in A_{260}) were pooled to give 55 ml of affinity-purified HSVTK. This material was concentrated by precipitation with 50 percent saturated ammonium sulfate and resuspended in 5 ml buffer A and stored at -20°C.

RESULTS

The preparation of 3′-amino-thymidine from commercially available 3′-azido-thymidine (AZT) by a modification of the procedure of Lin and Prusoff [11] gave an 85 percent yield of the AMT product. This product was identified by its chromatographic behavior in comparison to an authentic sample purchased commercially. The procedure is simple, can be carried out with minimal laboratory skill and equipment, and yielded a product which was suitable for linking to the affinity matrix without further purification. This simple synthesis gave the desired AMT at a considerable lower total cost than commercially available AMT. The coupling to the matrix followed the standard methods supplied by the manufacturer of the sepharose with 6-carbon carboxy-terminated linker arms.

To test the efficiency of the 3′-amino thymidine resin for the purification of thymidine kinases and to compare it with the thymidine p-aminophenyl phosphate resin, small columns were prepared from each resin and samples of purified herpes simplex virus type

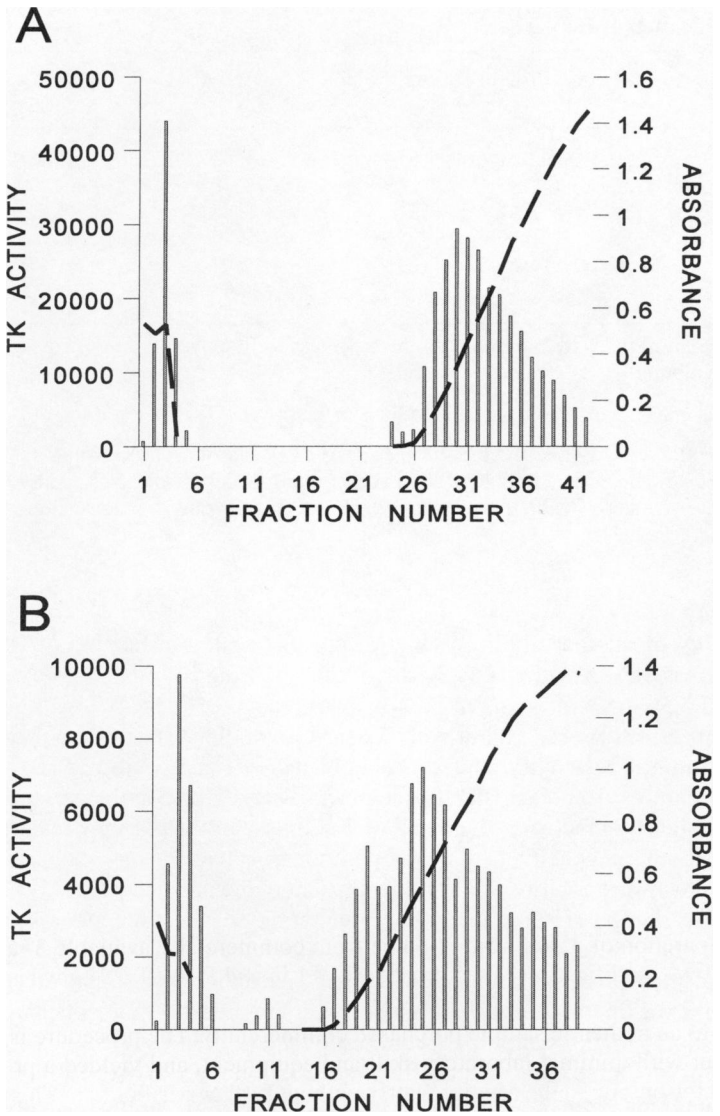

Figure 2. Chromatography of pure HSV TK on AMT resin (A) and thymidine p-aminophenyl phosphate resin (B). Residual thymidine in the applied sample resulted in the failure of the column to retain the initial portion of the sample. The gradient of thymidine concentration, from 0 to 200 micromolar, was monitored by optical absorbance at 260 nm (broken line). The TK activity was monitored by standard enzyme assays (vertical bars).

1 thymidine kinase [1] was applied to each column. The buffers and running conditions were the same for both resin. The elution profiles from two of these chromatographies are shown in Figure 2. Overall, the two resins behaved similarly with respect to the retention and elution of HSV-1 thymidine kinase. Gradient elution with increasing concentrations of thymidine showed that the affinities of the enzyme for the two resins were similar and that elution with 200 μM thymidine was sufficient to elute all the bound enzyme. The resin could be recycled for repeated use over at least a 12 month period.

Table 1. Purification of HSV TK

Fraction	Volume	Protein * Conc.	Total Protein	Activity ** (x 10^{-3})	Total Act. (x 10^{-3})	RSA†
Crude Ext	150 ml	11 mg/ml	1650 mg	15.8 U/ml	2,370	1.0
AS prec.	150	7.5	1120	13.8	2,070	1.3
DEAE FT	210	7.3	1530	5.8	1,220	0.6
AMT Load	75	13	975	27.9	2,090	1.5
AMT Pool	55	4.2	236	20.4	1,120	3.4
AMT Conc.	5	12	60	145	725	8.4

* Protein concentrations were measured by the Coomassie Blue dye-binding method (Biorad) with bovine serum albumin as a standard.

** Activity was measured in units as defined by Sanderson, et al. [1].

† Relative Specific Activity, i.e., the ratio of the specific activity of each fraction to that of the crude extract. It should be noted that both the activity and protein assays can be affected by the impurities present in the early steps of this purification; thus, the RSA is only a crude guide as to the fold-purification achieved.

The utility of this novel affinity matrix for large scale purification of HSVTK from bacterial cells is shown in Table 1. Starting with 1650 mg of total soluble protein in the crude extract, 236 mg of protein (14 percent) was recovered in the pooled fractions which were eluted from the AMT column with 0.2 mM thymidine. This pool contained 31 percent of the initial TK activity, and an 8.4 fold increase in specific activity of the TK. Analysis of aliquots from each step of the purification by SDS-polyacrylamide gel electrophoresis (Figure 3) indicates that the AMT affinity chromatography results in a purification almost to homogeneity. In some preparations, such as the one shown in Figure 3, a minor band of slower mobility was variably present in the pruified TK. This band was lost upon concentration by ammonium sulfate precipitation. This phenomenon may indicate that the minor band was a conformer of the TK or that the contaminant did not precipitate with the concentration of ammonium sulfate used. We did not investigate this problem further because it was inconstant and eliminated by ammonium sulfate concentration.

Analysis of the amino-terminal sequence of the protein by the Keck Laboratory at Yale School of Medicine indicated that the first 10 residues are ala-ser-tyr-pro-gly-his-gln-his-ala-ser-ala. This sequence corresponds to that predicted from the nucleotide sequence of the DNA starting with the residue immediately after the initial methionine. The concentrated protein at this stage of purification readily crystallizes from ammonium sulfate [27]. The crystallized protein is not appreciably more pure than the affinity column pool as determined by electrophoretic analysis or by specific activity measurements (Figure 4 and Table 1).

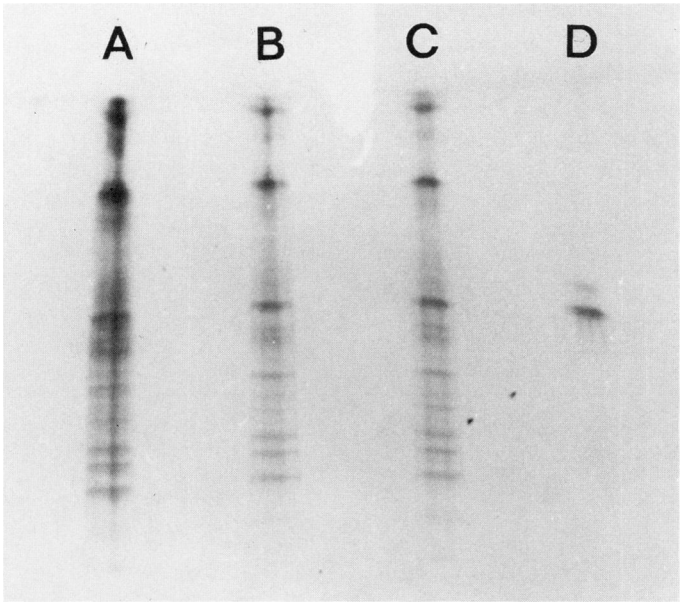

Figure 3. Analysis of the fractions from the large scale purification so HSV TK by SDS-polyacrylamide gel electrophoresis. Five microliter samples of each fraction (Table 1) was added to 5 microliter of SDS-containing gel sample buffer and heated for 3 min at 100°C. One microliter samples of this mixture were applied to each lane of this 7.5-15 percent polyacrylamide gel (pHastgel, Pharmacia) and subjected to electrophoresis. The gel is stained with Coomassie Brilliant Blue R. Lane A: crude extract. B: 50 percent ammonium sulfate precipitate. C: DEAE eluate. D: pooled AMT column eluate.

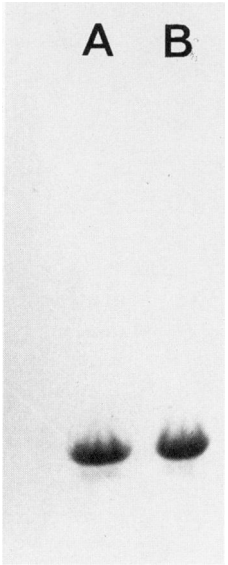

Figure 4. Gel electrophoresis of recrystallized TK (lane A); concentrated TK from the AMT column (lane B).

DISCUSSION

Among the several approaches reported for the affinity chromatography of thymidine kinases, the resin described by Kowal and Marcus appears to be the most widely used (Table 2). In our experience it has worked well. However, the recent unavailability of the starting material and the deterioration of the column after repeated use are two drawbacks to this material. The resin described in this paper performs as well as that of Kowal and Marcus and had the advantage of being simpler to prepare from readily available starting materials and of being resistant to hydrolytic degradation.

Table 2. Affinity columns for purification of TKs.

Column Ligand	References
3'-Amino-nitrophenyl Thymidine	Gan [11], Lai [12], Ellins [13, 14], Kit [15], Tamiya [16], Baron [17], Cheng [11], Sanderson [1], Eger [18, 19], Labenz [20]
AMP	Labenz [21]
Oligohistidine/metal	Franke [21]
Thymidine (epoxy coupled)	Grobner [22]
Glycoprotein inhibitor	Madhov [23]
5'-Amino-5-dideoxy thymidine	Rhode [24], Kit [16]
6-(p-Amino)benzyl-uracil (PABU)	Rhode [25]
Nucleotide tetraphosphate	Ikeda [26]

The use of the AMT resin on a preparative scale allows rapid and simple purification of HSV TK in large quantities from extracts of bacterial expression systems. The yield is high, the protein is pure and the product crystallizes. The use of a strain of *E. coli* deficient in bacterial thymidine kinase [5] eliminated the possibility that the bacterial TK might contaminate the HSV enzyme.

Acknowledgements: We thank Celine Melitz for supplying samples of pure TK, Margaret Black for supplying the plasmid pET8c:HSVTK. PPT was supported in part by grant T32 CA09159 from the National Institutes of Health.

REFERENCES

1. Sanderson, M.R., Freemont, P.S., Murthy, H.M.K., Krane, J.F., Summers, W.C., and Steitz, T.A. Purification and crystallization of thymidine kinase for herpes simplex virus type 1. J. Molec. Biol. 202:917-919, 1988.
2. Tung, P.P. and Summers, W.C. Substrate specificity of Epstein-Barr virus thymidine kinase. Antimicrob. Ag. Chemother. 38:2175-2179, 1994.
3. Kowal, E.P. and Markus, G. Affinity chromatography of thymidine kinase from a rat colon adenocarcinoma. Prep. Biochem. 6:369-385, 1976.
4. Sigma Chemical Co., St. Louis, MO, 1993 Catalog.
5. Summers, W.C. and Raksin, P. A method for selection of mutants at the *tdk* locus in *Escherichia coli*. J. Bacteriol. 175:6049-6051, 1994.
6. Black, Margaret. Personal communication: The HSVTK coding sequence bounded by NcoI restriction sites from pT7:HSVTK [7] was subcloned into the NcoI site of pET8c..
7. Black, M.E. and Hruby, D. A single amino acid substitution abolishes feed-back inhibition of vaccinia virus thymidine kinase. J. Biol. Chem. 267:9743-9748, 1992.

8. Summers, W.C. and Summers, W.P. ^{125}I-Deoxyuridine used in a rapid, sensitive, and specific assay for herpes simplex virus type 1 thymidine kinase. J. Virol. 24:314-318, 1977.
9. Lin, T.S. and Prusoff, W.H. Synthesis and biological activity of several amino analogues of thymidine. J. Med. Chem. 21:109-112, 1978.
10. Cheng, Y.C. Thymidine kinase from blast cells of myelocytic leukemia. Meth. Enzymol. 51:365-371, 1976.
11. Gan, T.E., Brumley, J.L., and Van der Weyden, M.B. Human thymidine kinase. Purification and properties of the cytosolic enzyme of placenta. J. Biol. Chem. 258:7000-7004, 1983.
12. Lai, M.H. and Weber, G. Increased concentration of thymidine kinase in rat hepatomas. Biochem. Biophys. Res. Comm. 111:280-287, 1983.
13. Ellims, P.H., Gan, T.E., and Cosgrove, L. Human thymidine kinase: purification and some properties of the TK1 isozyme from placenta. Molec. Cell. Biochem. 45:113-116, 1982.
14. Ellims, P.H. and Van der Weyden, M.B. Human liver thymidine kinase. Purification and some properties of the enzyme. J. Biol. Chem. 255:11290-11295, 1980.
15. Kit, S., Jorgensen, G.N., Dubbs, D.R., Trkula, D., and Zaslavsky, V. Detection of herpes simplex virus thymidine kinase polypeptides in cells labeled with ^{35}S-methionine. Intervirol. 9:162-172, 1978.
16. Tamiya, N., Yusa, T., Yamaguchi, Y., Tsukifuji, R., Kuroiwa, N., Moriyama, Y., and Fujimura, S. Co-purification of thymidylate kinase and cytosolic thymidine kinase from human term placenta by affinity chromatography. Biochim. Biophys. Acta 995:28-35, 1989.
17. Baron, G., Madec-Baron, A., Samperez, S., and Jouan, P. A rapid two-step purification of rat liver fetal thymidine kinase. Prep. Biochem. 20:241-256, 1990.
18. Eger, K., Klunder, E., Beck, R.A., and Scholz, U. Simplified synthesis of mono(4-aminophenyl)-3′-thymidylic acid ester used as thymidine kinase specific ligand for affinity chromatography. Pharmaceutical Res. 10:771-773, 1993.
19. Eger, K., Folkers, G., and Beck, R. Reinigungsmöglichkeiten von Thymidinkinasen mit Hilfe der Affinitätschromatographie. Pharmazie in Unserer Zeit 18:43-52, 1989.
20. Labenz, J., Friedrich, D., Mueller, W.E., and Falke, D. Adenosine- and ADP-phosphorylating capacity of herpes simplex virus-induced thymidine kinase enzyme complex. Intervirol. 19:77-84, 1983.
21. Franke, C.A. and Hruby, D.E. Expression and single-step purification of enzymatically active vaccinia thymidine kinase containing an engineered oligohistidine domain by immobilized metal affinity chromatography. Prot. Express. Purific. 4:101-109, 1993.
22. Grobner, P. and Loidl, P. Thymidine kinase. A novel affinity chromatography of the enzyme and its regulation in *Physarum polycephalum*. J. Biol. Chem. 259:8012-8014, 1984.
23. Madhov, R., Coetzee, M.L., and Ove, P. Purification of thymidine kinase by affinity chromatography with an enzyme inhibitor as the ligand. Arch. Biochem. Biophy. 200:99-107, 1980.
24. Rohde, W. and Lezius, A.G. The purification of thymidine kinase from *Escherichia coli* by affinity chromatography. Hoppe-Seyler's Zeits. Physiol. Chem. 352:1507-1516, 1971.
25. Rohde, W. A new affinity column for the purification of thymidine kinase from *Escherichia coli*. Anal. Biochem. 80:643-645, 1977.
26. Ikeda, S. and Ives, D.H. Multisubstrate analogs for deoxynucleoside kinases. Use in novel affinity media applied to resolution of *Lactobacillus* enzymes. J. Biol. Chem. 260:12659-12664, 1985.
27. Brown, D.G., Visse, R., Sandhu, G., Davies, A., Rizkallah, P.J., Melitz, C., Summers,. W.C., and Sanderson, M.R. Crystal structures of the thymidine kinase for herpes simplex virus type-1 in complex with deoxythymidine and Ganciclovir. Nature Struct. Biol. 2:876-881, 1995.