Pergamon

Tetrahedron: Asymmetry Vol. 6, No. 1, pp. 207-211, 1995

0957-4166/95 $9.50+0.00

0957-4166(94)00376-9

Differential Dye-Ligand Chromatography as a General Purification Protocol For 2-Keto-3-deoxy-6-phosphogluconate Aldolases.

Michael C. Shelton, and Eric J. Toone*

Department of Chemistry, P. M. Gross Chemical Laboratories, Duke University, Durham, NC 27708, USA

Abstract: 2-Keto-3-deoxy-6-phosphogluconate aldolases (KDPG aldolases, EC 4.1.2.14) from four sources, *Escherichia coli*, *Pseudomonas putida*, *Pseudomonas saccharophila*, and *Zymononas mobilis*, were purified from 17- to 48- fold by differential dye-ligand chromatography (DD-L chromatography). This technique, using Procion Navy H-ER (150 grain) as a negative column and Procion Yellow MX-GR as a positive column supported on Sepharose CL-4B, represents a general methodology for the rapid, facile purification of KDPG aldolases from disparate microbial sources.

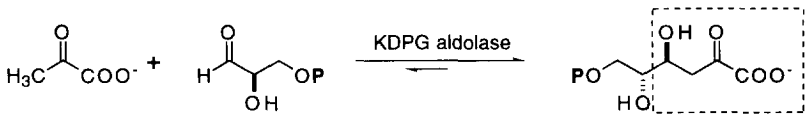
Enzymes are proven catalysts for myriad transformations in synthetic organic chemistry.[1-3] Aldolases are particularly valuable as catalysts for stereospecific carbon-carbon bond formation. To date, the bulk of synthetic aldolase chemistry has utilized the dihydroxyacetone phosphate aldolases.[4] Ultimately, the synthetic utility of this class of enzymes is limited by the conserved functionality produced by the DHAP aldolases: although the trihydroxybutanone produced is *densely* functionalized, it is not *differentially* functionalized.

Pyruvate aldolases are an attractive alternative group of synthetic catalysts. The 2-keto-4-hydroxybutanoate skeleton produced contains four different oxidation states of carbon in four contiguous carbons. This *differential* functionalization provides a synthetic advantage in comparison to DHAP aldolases. Although *N*-acetylneuraminic acid aldolase has been used extensively for the preparation of modified sialic acids,[1,5-7] the general utility of the pyruvate aldolases for stereocontrolled carbon-carbon bond formation has not yet been established.

2-Keto-3-deoxy-6-phosphogluconate aldolase (KDPG aldolase) is a central enzyme of the Entner-Doudoroff pathway of carbohydrate metabolism, and *in vivo* catalyzes the cleavage of 2-keto-3-deoxy-6-phosphogluconate (KDPG) to pyruvate and *D*-glyceraldehyde 3-phosphate.[8] Recently, we reported the synthetic utility of KDPG aldolase from *Pseudomonas putida*. Although the enzyme accepts a range of unnatural aldehydes as the electrophilic component and displays complete stereospecificity with unnatural substrates, not all aldehydes were accepted as substrates.[9] In order to fully develop the synthetic utility of pyruvate aldolases, we are investigating analogous aldolases from other bacterial sources.

Inattention to the pyruvate aldolases derives from two sources. First, the pyruvate aldolases are components of the Entner-Doudoroff (ED) pathway of carbohydrate metabolism, assumed to be absent from most organisms. More recent research has shown that many, if not most, organisms possess an ED pathway, even if it is not the primary route of carbohydrate metabolism.[10,11] Secondly, no general purification protocols exist for the isolation of ED pathway aldolases. We report here a rapid, general, facile procedure for the

isolation of pyruvate aldolases based on differential dye-ligand chromatography.



Scheme 1. KDPG aldolase-catalyzed condensation (the conserved 2-keto-4-hydroxybutyrate chain of formed in *all* KDPG aldolase-catalyzed condensations is enclosed in the box).

RESULTS & DISCUSSION

Differential dye-ligand (DD-L) chromatography exploits the serendipitous discovery that reactive dyes bind to proteins.[12] Dye columns act as "nonselective" affinity supports, and have been successfully used for the purification of numerous proteins and enzymes.[13,14] Typically, columns are used in pairs. A negative column removes undesired contaminants, but does not retain the desired protein. Following elution through the negative column, the protein of interest is retained by the positive column. The negative column is removed from the circuit, and the desired protein is eluted from the positive column.

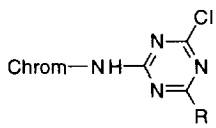


Figure 1. Triazine dye structure.

Dye columns were prepared according to a modified version of Lowe and Dean using triazine dyes.[13,14] The general structure of triazine dyes is shown in Figure 1. Procion Navy H-ER (150 grain) dye (Figure 1, R = arylamino) was used for the negative column and Procion Yellow MX-GR (Figure 1, R = Cl) served as the positive column. In both cases, Sepharose CL-4B was utilized as the solid support.[15] The halogen of the dye is displaced by a free hydroxyl on the polymeric support under slightly basic conditions, yielding fully immobilized dyes. We attempted to increase flow rates through the dye columns by utilizing Cellufine GC-700 as the polymeric support: this support permits the use of a peristaltic pump. Unfortunately, the aldolase was not retained on the columns, and the support was abandoned.

Crude aldolase from four sources, *E. coli, Zymononas mobilis, Pseudomonas putida* and *Pseudomonas saccharophila*, were loaded onto the columns. In the development of a general method for the purification of KDPG aldolases it was preferable to treat the cellular extracts in the same manner whenever possible. The crude extracts of all four aldolases were prepared by sonication of the cells, treatment with protamine sulfate, and ammonium sulfate fractionation. The partially purified extracts were passed first through the Procion Navy column followed by the Procion Yellow column. The columns were washed with phosphate buffer and the Procion Yellow column was eluted with racemic α-glycerophosphate. KDPG aldolase is then eluted from the Procion Yellow column with pyruvate and racemic α-glycerophosphate. Proteins can be eluted from dye columns by several methods.[16] We investigated two techniques for the desorption of KDPG aldolases: (1) nonselective elution by an increase in the ionic strength of the eluting buffer and (2) selective elution with a

substrate mimic. As expected, selective elution afforded aldolase with higher specific activity than that from nonselective elution. KDPG aldolase from *Pseudomonas putida* was obtained with a specific activity of 11.77 U mg^{-1} *via* selective elution with 20 mM pyruvate in 30 mM α-*DL*-glycerophosphate buffer while a specific activity of 1.21 U mg^{-1} was realized through nonspecific elution with 1 M NaCl.

Because leaching from dye columns is a problem frequently encountered in differential dye-ligand (DD-L) chromatography, we tested the feasibility of regenerating dye columns. The Procion Yellow MX-GR column can be successfully regenerated under the same conditions used for the initial binding to the solid support.

Table 1. Partial Purification of KDPG aldolases *via* DD-L Chromatography

Source	Initial Specific Activity	Final Specific Activity	Purification	Percent Recovery
Z. mobilis	17.89 U/mg	479.30 U/mg	26.79	114.9 %
E. coli	13.53 U/mg	407.32 U/mg	30.10	107.5 %
P. putida	0.25 U/mg	11.77 U/mg	47.85	70.2 %
P. saccharophila	1.85 U/mg	32.27 U/mg	17.42	71.6 %

Table 1 shows that differential dye-ligand chromatography is a general method for the partial purification of KDPG aldolases from various sources. 17- to 48- fold purifications are attained in less than one day total working time with virtually no loss in total activity. SDS-PAGE gels of both *E. coli* and *Zymononas* aldolases show single bands, suggesting purities of >95%. Purifications of some pyruvate aldolases have been documented, however all are tedious and provide low recovered yields.[1,5-7,17-20] The success of differential dye-ligand chromatography for the purification of the *Z. mobilis* KDPG aldolase[15] coupled with the extensive sequence homology between KDPG aldolases[10] led us to investigate DD-L chromatography as a general method for the purification of KDPG aldolases. The amino acid sequences of KDPG aldolases from *E. coli*, *P. putida* and *Z. mobilis* have been previously determined and contain numerous sequence homologies, in particular near the active site.[10] The observed sequence homology, in particular the similarity of the active sites may be the basis of the generality of DD-L chromatography for the purification of these aldolases.

We are currently evaluating these aldolases as synthetic catalysts and will report our results in due course.

EXPERIMENTAL

General procedures. Protein concentrations were determined by the method of Layne or a modified Lowry method.[21,22] Kinetic assays were performed on a Hewlett-Packard HP-8452A spectrophotometer. The Procion dyes were a gift of ICI Zeneca Colors, Inc. Charlotte, NC. Glucose 6-phosphate (sodium salt), *L*-lactic dehydrogenase (EC 1.1.1.27, Type II from rabbit muscle), NADH and Sepharose CL-4B were purchased from Sigma Chemicals. Plasmids pTC 162 and pTC 190, encoding KDPG aldolases from *Zymononas mobilis* and *E. coli* respectively, were generous gifts of T. Conway, School of Biological Sciences, University of Nebraska.[23,24] *E. coli* strain DF214[25] was a gift of B. Bachmann, *E. Coli* Genetic Stock Center, Yale University. Plasmid transformation into E. coli was performed according to the method of Silhavy.[26]

Assay for KDPG aldolase. KDPG aldolase (EC 4.1.2.14) activity was determined using a coupled assay with *L*-lactic dehydrogenase (EC 1.1.1.27, from rabbit muscle). The following conditions were employed. 50 mM KH_2PO_4, pH 7.5 buffer (3.0 μL) was added to a plastic cuvette. KDPG aldolase (5 μL: pTC 190 and pTC 162 and 10 - 50 μL *P. putida* and *P. saccharophila*) along with NADH (50 μL, 15 mg/mL) were added to the cuvette. KDPG-Li_2^+ (100 μL, 100 mg/mL) was then added to the assay mixture. Enzyme activity was determined from the diappearance of NADH.

Purification of KDPG aldolase from *E. coli* (pTC 190). *E. coli* (DF214{pTC190}) was grown on Luria broth (Miller's modification) supplemented with 0.5 % potassium gluconate innoculum for 8 hours, followed by growth on the same medium in 4-L Erlenmeyer flasks at 37° C. The cells were harvested by centrifugation (19,690 x g, 10 min.) and then suspended in 20 mM KH_2PO_4, pH 6.5 augmented with 1 mM PMSF. The cells were disrupted by sonication with a Heat Systems Ultrasonics sonicator/cell disrupter (macro tip, power level 10) at 0° C for a total of five minutes. The resulting extract was treated with 2% protamine sulfate, pH 5.5 (2 volumes) for 10 to 15 minutes at room temperature. The mixture was then centrifuged (37,050 x g, 30 min.). The resulting supernatant was made to 25% saturation with solid $(NH_4)_2SO_4$. The solid was removed by centrifugation (37,050 x g, 20 min.). The supernatant was made to 50% saturation with solid $(NH_4)_2SO_4$. The solid was removed by centrifugation (37,050 x g, 20 min.). The supernatant was then made to 85% saturation with solid $(NH_4)_2SO_4$. The pellet following centrifugation (37,050 x g, 20 min.) was suspended in 20 mM KH_2PO_4, pH 6.5 and dialyzed overnight against 20 volumes of the same buffer with three buffer changes. The extract (1 g of protein maximum) was placed on the Procion Navy column in sequence with the Procion Yellow column. The Procion Navy column was washed with 20 mM KH_2PO_4, pH 6.5 (100 mL). The Procion Navy column was removed from the sequence and the Procion Yellow column was washed with 20 mM KH_2PO_4, pH 6.5 (100 mL). The Procion Yellow column was eluted with 30 mM α-*DL*-glycerophosphate, pH 6.5 by adding 50 mL of this buffer to the column. The aldolase was selectively eluted with 20 mM sodium pyruvate in 30 mM α-*DL*-glycerophosphate, pH 6.5 (100 mL). The aldolase was then dialyzed against 20 mM KH_2PO_4, pH 6.5 to remove the pyruvate and α-*DL*-glycerophosphate.

Purification of KDPG aldolase from *Zymononas mobilis*. The *Z. mobilis* aldolase was obtained from *E. coli* DF214 *eda*$^-$ (lacking the gene for KDPG aldolase) transformed with a plasmid containing the *eda* gene coding for KDPG aldolase in *Z. mobilis*.[24-26] The cells were grown under the same condition, and the extract was prepared as described above for the purification of KDPG aldolase from *E. coli*.

Purification of KDPG aldolase from *Pseudomonas saccharophila*. *Pseudomonas saccharophila* was grown on minimal medium with sucrose as the sole carbon source as described by DeLey.[27] The extracts were prepared as described above, with the following exception, 20 mM KH_2PO_4, pH 6.5 was used to suspend the cells. 1mM PMSF was not required.

Purification of KDPG aldolase from *Pseudomonas putida*. *Pseudomonas putida* was grown on minimal medium with potassium gluconate as the sole carbon source as described by Meloche.[19b] The extracts were prepared as described above for the purification of *P. saccharophila*, with the following exception. The

ammonium sulfate fractionations were performed at 25% and 65%, with the aldolase found in the 65% pellet.

REFERENCES.

1. Wong, C. -H.; Whitesides, G. M.; *Enzymes in Synthetic Organic Chemistry*, Elsevier Science, New York, **1994**.
2. Roberts, S. M. *Introduction to Biocatalysis Using Enzymes and Microorganisms* Cambridge University Press, New York, **1994**.
3. a. Santaniello, E.; Ferraboschi, P.; Grisenti, P.; Manzocchi, A. *Chem. Rev.* **1992**, *92*, 1071.
   b. Toone, E. J.; Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *Tetrahedron* **1989**, *45*, 5365.
   c. Drueckhammer, D. G.; Hennen, W. J.; Pederson, R. L.; Barbas, III, C. F.; Gautheron, C. M.; Krach, T.; Wong, C. -H. *Synthesis* **1991**, 499.
4. a. Wong, C. -H.; Whitesides, G. M. *J. Org. Chem.* **1983**, *48*, 3199.
   b. Durrwachter, J. R.; Drueckhammer, D. G.; Nozaki, K.; Sweers, H. M.; Wong, C. -H. *J. Am. Chem. Soc.* **1986**, *108*, 7812.
   c. Bednarski, M. D.; Simon, E. S.; Bischofberger, N.; Fessner, W. -D.; Kim, M. -J.; Lees, W.; Saito, T.; Waldmann, H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1989**, *111*, 627.
   d. Straub, A.; Effenberger, F.; Fischer, P. *J. Org. Chem.* **1990**, *55*, 3926.
   e. Hung, R. R.; Straub, J. A.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 3849.
   f. Liu, K. K. -C.; Kajimoto, T.; Chen, L.; Zhong, Z.; Ichikawa, Y.; Wong, C. -H. *J. Org. Chem.* **1991**, *56*, 6280.
   g. Look, G. C.; Fotsch, C. H.; Wong, C. -H. *Acc. Chem. Res.* **1993**, *26*, 182.
5. Kim, M. -J.; Hennen, W. J.; Sweers, M.; Wong, C. -H. *J. Am. Chem. Soc.* **1988**, *110*, 6481.
6. Augé, C.; David, S.; Gautheron, C. *Tetrahedron Lett.* **1984**, *25*, 4663.
7. Bednarski, M. D.; Chenault, H. K.; Simon, E. S.; Whitesides, G. M. *J. Am. Chem. Soc.* **1987**, *109*, 1283.
8. Entner, N.; Doudoroff, M. *J. Biol. Chem.* **1952**, *196*, 853.
9. Allen, S. T.; Heintzelman, G. R.; Toone, E. J. *J. Org. Chem.* **1992**, *57*, 426.
10. Conway, T. *FEMS Microbiol. Rev.* **1992**, *103*, 1 and references therein.
11. Lessie, T. G.; Phibbs, P. V. *Ann. Rev. Microbiol.* **1984**, *38*, 359.
12. Haeckel, R.; Hess, B.; Lauterborn, W.; Wruster, K. *Hoppe-Seyler's Z. Physiol. Chem.* **1968**, *349*, 699.
13. Lowe, C. R.; Pearson, J. C. *Met. Enzymol.* **1984**, *104*, 97.
14. Dean, P. D. G.; Watson, D. H. *J. Chromatog.* **1979**, *165*, 301.
15. Scopes, R. K. *Anal. Biochem.* **1984**, *136*, 525.
16. Scopes, R. K. *Protein Purification: Principles and Practice*; 3rd ed. Springer-Verlag: New York, **1994**; p 210-226.
17. Floyd, N. C.; Liebster, M. H.; Turner, N. J. *J. Chem. Soc. Perkin Trans. 1* **1992**, 1085.
18. Sugai, T. Shen, G. -J.; Ichikawa, Y.; Wong, C. -H. *J. Am. Chem. Soc.* **1993**, *115*, 413.
19. a. Meloche, H. P.; Wood, W. A. *J. Biol. Chem.* **1964**, *239*, 3515.
   b. Meloche, H. P.; Ingram, J. M.; Wood, W. A. *Method. Enzymol.* **1966**, *IX*, 520.
   c. Hammerstedt, R. H.; Mohler, H.; Decker, K. A.; Wood, W. A. *J. Biol. Chem.* **1971**, *246*, 2069.
20. Meloche, H. P.; O'Connell, E. L. *Method. Enzymol.* **1982**, *90*, 263.
21. Layne, E. *Method. Enzymol.* **1957**, *3*, 447.
22. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
23. Egan, S. E.; Rliege, R.; Tong, S.; Shibata, A.; Wolf, R. E., Jr.; Conway, T. *J. Bacteriol.* **1992**, *174*, 4638.
24. Conway, T.; Fliege, R.; Jones-Kilpatrick, D.; Liu, J.; Barnell, W. O.; Egan, S. E. *Molec. Microbiol.* **1991**, *5*, 2901.
25. Vinopal, R. T.; Hillman, J. D.; Schulman, H.; Reznikoff, W. S.; Fraenkel, D. G. *J. Bacteriol.* **1975**, *122*, 1172.
26. Silhavy, T. J.; Berman, M. L.; Enquist, L. W. *Experiments with Gene Fusion*, Cold Spring Harbor Laboratories, **1984**, 169.
27. DeLey, J.; Doudoroff, M. *J. Biol. Chem.* **1957**, *227*, 745.

Acknowledgements. The financial support of the National Institutes of Health (GM 481841) and the Petroleum Research Fund, administered by the ACS (27330-AC4) is greatfully acknowledged.

(*Received in USA* 29 *September* 1994)