ENZYME PURIFICATION BY SELECTIVE ELUTION WITH SUBSTRATE FROM SUBSTITUTED CELLULOSE COLUMNS

Burton M. Pogell, Department of Microbiology, Vanderbilt University School of Medicine, Nashville 5, Tennessee

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The most unique and distinguishing properties of the enzyme proteins are those expressed in their substrate specificities. Very little advantage has been taken of these specific substrate affinities in enzyme purification. Known examples include (a) purification of α-amylase and separation of α-amylase from β-amylase by adsorption on "insoluble" starch and elution with "soluble" starch (1), (b) purification of glycogen synthetase by isolation of liver particulate glycogen, to which the enzyme is bound (2), (c) the association of starch synthetase with bean starch granules (3), and (d) elution of yeast pyrophosphatase from Cγ alumina gel with dilute pyrophosphate (4). The purpose of this communication is to report the attainment of very high purifications of fructose-1,6-diphosphatase (FDPase) and aldolase by stepwise elution from CM-cellulose columns with dilute solutions of fructose-1,6-diphosphate (FDP) and to give evidence for the specificity of this elution technique.

The general procedure employed in the presented experiments was as follows: 0.5-0.6 g of CM-cellulose (standard Selectacel, C. Schleicher and Schuell Co., Keene, New Hampshire) was suspended in 0.005 M sodium malonate (pH 6.0) and the pH readjusted to 6.0 by NaOH addition. Columns were prepared by pouring the slurry without pressure into tubes of 1.2 cm inside diameter with glass wool placed at both the bottom and top of the cellu

lose. All column experiments were carried out at 0-4°. Flow rates were about 3 ml per minute, but this did not appear to be critical. The amount of initial extract necessary to saturate the column with the particular enzyme under study was determined experimentally. This usually was chosen so that 5 to 10% of the applied enzyme was not retained on the cellulose. Next the column was washed with malonate until there was no protein in the effluent as measured by the absorbancy difference ($A_{215m\mu}$-$A_{225m\mu}$) with malonate as blank (5). This was followed by elution with 0.005 M sodium malonate containing the specific eluent being tested and at pH 6.0. Specific activity values for both enzymes are given in μmoles of product formed per hour per mg of protein.

Results of two different sets of experiments with FDPase are summarized in Table I. In Part A, the starting material was rabbit liver enzyme which had been partially purified by high-speed centrifugation to remove particulate material, acid precipitation to remove further insoluble protein, and ammonium sulfate fractionation followed by dialysis and lyophilization. The enzyme had a specific activity of 12.5, representing about a 12.5 fold purification of the crude homogenate. The minimum NaCl concentration which eluted greater than 90% of the FDPase was found to be 15 mM and gave material of specific activity around 100. In contrast, concentrations of either the dimagnesium or sodium salts of FDP in the range of 0.05 to 0.15 mM eluted the bulk of the enzyme activity with much lower amounts of contaminating protein. Since a divalent metal is necessary for phosphatase activity, elution by the sodium salt either indicates the presence of small amounts of magnesium in the enzyme preparations or interaction of the substrate and enzyme in the absence of divalent metal. The specificity of the FDP elution was shown by the fact that other salts, including 10 fold higher concentrations of the hexose monophosphates, only partially removed the enzyme from the column

TABLE I

Specificity of elution of fructose-1,6-diphosphatase from CM-cellulose columns

FDPase activity was determined at 38° as previously described (6) with 0.01 M mercaptoethanol replacing 0.005 M cysteine. All eluents were dissolved in 0.005 M sodium malonate (pH 6.0) and the pH readjusted to pH 6.0. Where more than one eluent is listed in a single experiment, the respective solutions were applied in the order shown. Part A. 10 mg of lyophilized sample (specific activity of 12.5) was dissolved in 1 ml of malonate buffer and applied to a 0.6 g CM-cellulose column. At least 5-10 ml fractions were collected before changing to the below eluents. Protein was determined by either the 280 mμ absorbancy or the absorbancy difference at 215 mμ and 225 mμ (5). In experiments 1 and 2, 20 mg of lyophilized sample was applied to a 1.2 g column. Part B. 31 mg of lyophilized sample (specific activity of 5.7 to 6.1 based on protein content and 3.5 to 3.7 on a dry weight basis) was dissolved in 1 ml of malonate buffer and applied to a 0.5 g CM-cellulose column. The column then was washed with buffer til the absorbancy difference ($A_{215m\mu}$-$A_{225m\mu}$) was zero. This took from 150 to 200 ml. Protein was determined by the micro Lowry procedure (7).

Experiment		Eluent	Concentration (mM)	Per Cent Eluted	Specific Activity	Purification Factor
A.	1	NaCl	15	83	114	9
	2	Mg_2FDP	0.14	97	818	65
	3	Na_4FDP	0.05	80	1,040	83
	4a	$MgCl_2$	20	0	—	—
	b	"	100	70	31	5.5
	5	Na_2F6P	0.46	51	123	9.8
	6a	Na_2G6P	0.50	18	157	12.6
	b	K_2G1P	0.50	4	—	—
	c	Na_4FDP	0.05	36	102	8.1
B.	1	Mg_2FDP	0.05	37	1,770	290
	2	Na_4FDP	0.15	58	935	153
	3	"	0.30	90	692	122
	4a	Na_2G6P	0.30	0	—	—
	b	"	3.0	>87	446	79
	5a	K_2G1P	0.30	0	—	—
	b	"	3.0	38	190	32
	c	Na_4FDP	0.30	24	437	74

and gave much lower specific activities. In Part B, the starting material was prepared by dialyzing a high-speed supernatant and removing any precipitate formed on adjustment to pH 6.0. The capacity of the CM-cellulose for FDPase with this preparation was about the same as that found with the purer

starting material. FDP eluted most of the enzyme in a sharp peak, whereas the activity came off the column in a much more diffuse manner with either 3 mM glucose 6-phosphate or glucose 1-phosphate. Two maxima were observed with these latter eluents, similar to that found with aldolase (see below). It should be noted, as seen in Exp. 6c of Part A and Exp. 5c of Part B, that although elution with FDP after hexose monophosphate application removed a large percentage of the remaining enzyme, the specific activity was much lower than that found by elution with FDP alone. In this type of column experiment, pretreatment with any eluent apparently alters the effect of the second eluent.

Illustrative elution patterns found with rabbit liver aldolase are shown in Figures 1a and b. The minimum sodium FDP concentration necessary to elute greater than 90% of the adsorbed enzyme at pH 6.0 was found to be 2.5 mM. The peak fraction contained enzyme 29 fold purer than the initial material. Specific activities as high as 44 at 24° have been obtained in other experiments, which may be compared with the reported value of 60 found at 25° with crystalline bovine liver aldolase assayed under almost identical conditions (9). With 30 mM NaCl as eluent, the aldolase was eluted in a much more diffuse band and with much less purification. Most of the aldolase was separated from the FDPase in the CM-cellulose columns, as would be predicted from the difference in FDP concentrations necessary to elute these enzymes.

Purifications obtained with this procedure represent a different order of magnitude over those found with classical fractionation steps and permit quantitative recovery of the enzyme. Experiments now are in progress to test the generality of this method, its usefulness in separating different

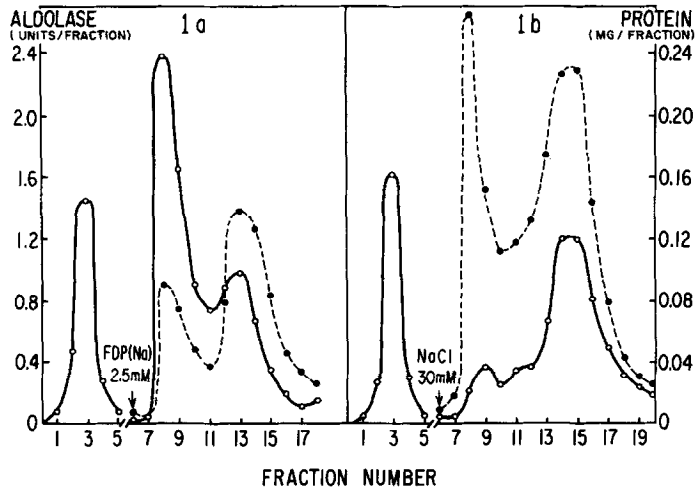

Figure 1a and b. Elution patterns of aldolase from CM-cellulose columns.

1.6 ml of dialyzed rabbit liver high-speed supernatant, adjusted to pH 6.0 and centrifuged to remove the precipitate, was applied to a 0.5 g CM-cellulose column. After washing the column with 115 ml of malonate buffer, the eluents shown on the graphs were applied. Aldolase activity was determined as described by Wu and Racker (8). In fractions containing FDP, where some aldolase action may have occurred at 0°, readings were taken after reaching linearity of absorbancy decrease at 340 mμ with time. Protein was determined by the micro Lowry method (7). The initial specific activity in Fig. 1a was 0.91 and 27° and in Fig. 1b, 0.73 at 23°. 70% of the protein and 19-24% of the aldolase were in the first five fractions of both experiments. Overall recovery of enzyme was greater than 99%. Fractions 6 to 18 in Fig. 1a were each 2.7 ml and all other fractions were 3.1 ml. O——O, aldolase; ●------●, protein.

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enzymes from crude tissue extracts, and what relation the selective elution may have to the formation of enzyme-substrate complexes.

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