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AFFINITY CHROMATOGRAPHY OF β-GALACTOSIDASE ON CONTROLLED-PORE GLASS DERIVATIVES

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SUMMARY

Several controlled-pore glass (CPG) derivatives were examined as supports for the affinity chromatographic purification of the enzyme β-galactosidase. The competitive inhibitor *p*-aminophenyl-β-D-thiogalactopyranoside was coupled to an azelaic acid and a malonic acid derivative of 750-Å alkylamine CPG of 80–120 mesh and to an azelaic acid derivative of 550-Å alkylamine CPG of 40–80 mesh. The latter derivative exhibited particularly good load capacity and separation efficiency; however, both arm lengths were effective. Hydrophobic interactions between the arm and the enzyme contribute to the separation.

INTRODUCTION

Affinity chromatography has been demonstrated to be an efficient technique for the purification of enzymes and other biological materials[1]. The purification of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) by affinity chromatography on cross-linked bovine γ-globulin[2], agarose[3–5] and controlled-pore glass (CPG)[6] has been reported. In the latter report, the affinity ligand *p*-aminophenyl-β-D-thiogalactopyranoside (PAPTG) was coupled to 750-Å CPG of 120–200 mesh size by the diazonium chloride intermediate.

A wide variety of CPG products is available and numerous coupling reactions can be employed[7]. We have briefly examined the role of various carrier properties on the separation process employing PAPTG coupled to two dicarboxylic acid derivatives of alkylamine CPG.

METHODS

Materials

Alkylamine CPG, 550 Å, 20–80 mesh, was obtained from Pierce (Rockford, Ill., U.S.A.), the 750-Å alkylamine CPG of 80–120 mesh was kindly provided by Dr. M. Lynn and D. Eaton of Corning (Corning, N.Y., U.S.A.).

p-Nitrophenyl-β-D-galactopyranoside and PAPTG were purchased from Calbiochem. (Los Angeles, Calif., U.S.A.). The β-D-galactosidase (*Aspergillus niger* L.P.)

was purchased from Wallerstein Labs. (Deerfield, Ill., U.S.A.). The dibasic acids were reagent grade chemicals from Eastman-Kodak (Rochester, N.Y., U.S.A.).

All columns had an inside diameter of 6 mm. The packed column lengths of 1 g of 40–80 mesh and 80–120 mesh glass were 9.5 cm and 4.5 cm, respectively.

Preparation of carboxylic acid derivatives

A mixture of 10 g of alkylamine CPG, 0.02 moles of malonic or azelaic acid and 1.0 g of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfate (CDI; Aldrich, Milwaukee, Wisc., U.S.A.) in 75 ml of distilled water was placed on a shaker bath at 60° for 16 h. The supernate was decanted and the glass particles were washed with absolute methanol and water until the wash was neutral.

Coupling of the inhibitor to carboxylic acid-derivatized glass

One gram of the carboxylic acid-derivatized glass is added to a solution of 0.250 g of PAPTG and 0.40 g CDI in 20 ml of distilled water. The pH is adjusted frequently to 4.7 for the first hour and the mixture is then shaken at 22° for 16 h. The glass particles are then packed into a column 0.6 × 4.5 cm and washed continuously with 0.1 *M* borate buffer pH 7.5 until the eluate exhibits no absorbance at 280 nm.

Enzyme assay

The enzyme was assayed by incubating 0.25 ml of the enzyme solution with 0.1 ml of 8.3 m*M* *o*-nitrophenol-*β*-galactopyranoside in 0.1 *M* sodium acetate buffer pH 4.0 at 60° for 15 min. The reaction is terminated with 1.0 ml of a 10% sodium carbonate solution. The liberated *p*-nitrophenol is determined at 420 nm. One unit of activity is the quantity required to release 1 *μ*mole of *o*-nitrophenol per minute under the above assay conditions.

RESULTS AND DISCUSSION

We initially repeated the experiments of Steers *et al.*[3] using derivative C. This derivative is prepared by coupling PAPTG to the 3-succinyl-3'-aminodipropylamine derivative of Sepharose 4B. Our results were essentially similar to the reported work. We did use a different enzyme source (*A. niger*) rather than the *Escherichia coli* and some differences in binding characteristics may be expected (see Table I). About one fifth of the applied activity passed through without retention. We recovered about one half of the applied activity in the borate fraction. About 80% of the total applied activity was recovered.

In Table II we show a typical separation with the 750-Å CPG of 80–120 mesh with PAPTG coupled through an azelaic acid bridge. A column of nearly half the volume of the Sepharose column allowed the separation of nearly twice the amount of purified enzyme. A much higher degree of ligand substitution is possible on CPG than on agarose. About 6 *μ*moles of PAPTG can be coupled per ml of packed Sepharose 4B compared to about 50 *μ*moles of PAPTG per g of CPG based on a titration of the unsubstituted carboxyl groups. Only 60% of the applied enzymatic activity and protein were recovered in the CPG column. In subsequent experiments, it was found that essentially quantitative recovery of enzymatic activity can be obtained at lower column loadings.

TABLE I

SEPARATION OF *A. NIGER* β-GALACTOSIDASE ON A SEPHAROSE PAPTG COLUMN

Column dimensions, 8 × 0.9 cm; column temperature, 22°; flow-rate, 0.865 ml/min; enzyme in column 80 min before elution started. Added to column: 0.5 ml of a solution containing 40 mg solids/ml, 530 E.U./ml, and 2.35 mg protein.

Fraction No.	*Volume (ml)*	*Buffer*	*E.U. eluted*	*Recovered (%)*	*Specific activity (E.U./mg)*	*Protein (mg)*
1	5.1	Tris, pH 7.5	38	14.6	45	0.82
2	5.0	Tris, pH 7.5	16.8	6.4	36	0.45
3	5.1	Tris, pH 7.5	4.1	1.5	25	0.15
4	5.1	Tris, pH 7.5	1.32	0.5	16	0.08
5	14.5	Tris, pH 7.5	2.78	1.0	14.8	0.18
6	6.8	Borate, pH 10.0	—	—	—	—
7	3.3	Borate, pH 10.0	12.0	4.5	165	0.07
8	2.0	Borate, pH 10.0	117.6	44	321	0.36
9	9.8	Borate, pH 10.0	15.9	6.0	148	0.11
10	16.3	Borate, pH 10.0	1 i	0.4	—	—
			Total	78.9		2.22

TABLE II

TYPICAL SEPARATION ON A 750-Å AZELAIC ACID COLUMN

Support, 1 g of 750-Å alkylamine CPG of 80–120 mesh; flow-rate, 0.5 ml/min; column temperature, 22°. Added to column: 1.0 ml of a solution containing 40 mg solids/ml, 472 E.U./ml, and 4.65 mg protein. Derivative: azelaic acid–thiogalactose.

Fraction No.	*Volume (ml)*	*Buffer*	*E.U. eluted*	*Recovered (%)*	*Specific activity (E.U./mg)*	*Protein (mg)*
1	1.4	Tris, pH 7.5	8.4	1.7	58.9	0.143
2	3.4	Tris, pH 7.5	37.9	8.0	35.8	1.04
3	6.8	Tris, pH 7.5	40.8	8.6	60.7	0.67
4	5.3	Tris, pH 7.5	—	—	—	—
5	3.7	Borate, pH 10.0	195	41.3	224	0.87
			Total	59.6		2.723 (58.5%)

The length of the "arm" between the affinity ligand and the carrier is reported to have a major effect on the strength of the binding between the protein and the ligand. In earlier studies[3] with Sepharose, the ligand at the end of a short arm (10 Å) only slightly retarded β-galactosidase; however, with a longer arm (21 Å), it is necessary to use 0.01 *M* borate buffer at pH 10.0 to elute the enzyme. Woychik and Wondolowski[6] interposed an arm of about 17-Å length ($-OSi(CH_2)_3NHCOC_6H_4NH-$) and were able to remove the bound enzyme at pH 8.0 with a pyrophosphate buffer. We employed an alternative reaction which permitted facile preparation of derivatives of varying arm lengths. The CPG alkylamine derivative readily reacts with dibasic acids to yield a carboxyl end group which in turn readily reacts with the affinity ligand:

$$CPG\text{–}(CH_2)_3NH_2 + HOOC(CH_2)_nCOOH \rightarrow CPG\text{–}(CH_2)_3NHCO(CH_2)_nCOOH + H_2O$$

TABLE III

BINDING CAPACITY OF THE 750-Å AZELAIC ACID DERIVATIVE

Support, 1 g of 750-Å alkylamine CPG of 80–120 mesh; flow-rate, 2 ml/min; column temperature, 22°. Derivative: azelaic acid–thiogalactose. Given values are from single or duplicate runs.

E.U. added	*Borate fraction*		
	E.U.	*Specific activity*	*% Recovered*
425	135	176	32
607	224	185	35
848	211, 230	188, 150	25, 27
1213	237	185	19.5

TABLE IV

TEMPERATURE STUDIES WITH THE 750-Å MALONIC ACID DERIVATIVE

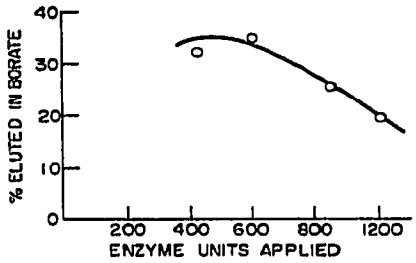
Support, 1 g of 750-Å alkylamine CPG of 80–120 mesh. Added to column: 0.50 ml of a solution containing 967 E.U./ml. Derivative: malonic acid–thiogalactose. Given values are from single or duplicate runs.

Temperature (°C)	*Flow-rate (ml/min)*	*Recovered in borate fraction*		
		E.U.	*%*	*Specific activity*
37	1.0	205, 274	20, 28	297, 364
22	1.0	208, 231	21, 24	—
10	1.0	131, 83	14, 8.6	249, 142
10	0.5	221, 188	23, 19	179, 234

Using 750-Å CPG, we prepared both the malonic acid $HOOC(CH_2)COOH$ and azelaic acid $HOOC(CH_2)_7COOH$ derivatives. The total arm length of the malonic acid derivative is about 12 Å, the arm containing azelaic acid is almost twice as long. Surprisingly, there was little difference between the binding tenacity of these two products (Tables III and IV). The enzyme eluted easily with 0.1 *M* borate buffer, pH 7.5, but not with 0.1 *M* Tris, pH 7.5. Both columns retained about 1 mg protein per gram of glass (20–30% of applied activity). At 2 ml/min elution the same capacity can be maintained; however, the per cent retained is related to the amount of protein applied (Figs. 1 and 2).

The specific activity of the eluted fractions was also equivalent. The relative binding strength of these two derivatives can be determined using the gradient elution techniques of Lowe *et al.*[8]. Active enzyme was eluted in both the Tris and borate fractions. The columns were operated at three temperatures, *viz.* 37°, 22° and 10°. At 10° the percent enzyme recovered in the borate fraction was substantially less than at the higher temperatures. At 37° and 22° the binding capacity of the columns are about equivalent (Tables IV and V).

As a control column, we used aniline in place of the inhibitor on the azelaic acid derivative. Active enzyme was obtained in both the Tris and borate fractions. The specific activity of the borate-eluted fraction was about 75% of the activity routinely obtained with the PAPTG columns. At low loading the column exhibited fair retention; however, at high loading very low retention is observed (see Table VI).



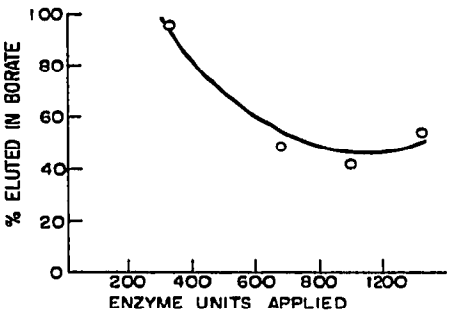


Fig. 1. Graph showing the recovery of enzyme in borate *vs.* the amount of enzyme applied. Support, 1 g of 750-Å alkylamine CPG of 80–120 mesh; flow-rate, 2 ml/min; column temperature, 22°. Derivative: azelaic acid–thiogalactose.

Fig. 2. Graph showing the recovery of enzyme in borate *vs.* the amount of enzyme applied. Support, 1 g of 550-Å alkylamine CPG of 40–80 mesh; flow-rate, 1 ml/min; column temperature, 22°. Derivative: azelaic acid–thiogalactose.

TABLE V

FLOW-RATE AND TEMPERATURE STUDIES WITH THE 750-Å AZELAIC ACID DERIVATIVE

Support, 1 g of 750-Å alkylamine CPG of 80–120 mesh. Derivative: azelaic acid–thiogalactose. Given values are from single or duplicate runs.

E.U. added	*Temperature (°C)*	*Flow-rate (ml/min)*	*Recovered in borate fraction*		
			E.U.	*Specific activity*	*%*
1295	22	0.5	398, 248	187, 163	31, 19
1295	22	1.0	333, 262	167, 177	26, 20
1295	22	2.0	248, 222	235, 213	19, 17
1885	37	1.0	240, 240	200, 202	10, 11
1885	10	1.0	13	296	0.7

TABLE VI

SEPARATION ON A 750-Å CONTROL COLUMN

Support, 1 g of 750-Å alkylamine CPG of 80–120 mesh; flow-rate, 1 ml/min; column temperature, 22°. Derivative: azelaic acid–aniline. Given values are from single or duplicate runs.

E.U. added	*Recovered*				
	Tris fraction		*Borate fraction*		
	E.U.	*Specific activity*	*E.U.*	*Specific activity*	*%*
750	644, 610	73, 75	128, 98	151, 143	17, 14
1500	1490, 1525	87, 88	28, 31	18(?), 175	2, 2

These results suggest the occurrence of binding to other elements of the PAPTG column. Possibly hydrophobic interactions occur with the long methylene bridge of azelaic acid and the enzyme. Hofstee[9] has reported the binding of several enzymes to agarose columns to which long-chain alkylamines were coupled by the cyanogen bromide method.

The 550-Å, 40–80 mesh column with an azelaic acid "arm" yielded very satisfactory results. The larger particle size was much more convenient to use and we did not encounter any plugging. The capacity of the column was about 2.5 mg enzyme per gram of glass. At low loading (1.5 mg enzyme per gram of glass) the borate fraction contained essentially all of the added enzyme (Fig. 2). A four times increase in loading did not drop the binding capacity of the column below 50% (see Table VII). This preparation had about twice the binding capacity of the 750-Å column.

Optimization and comparison of various CPG derivatives presented several difficulties. The observed percent retention was influenced by flow-rate, loading, pore diameter and even mesh size (a 1-g column of 40–80 mesh glass is nearly twice as long

TABLE VII

BINDING CAPACITY OF THE 550-Å AZELAIC ACID DERIVATIVE

Support, 1 g of 550-Å alkylamine CPG of 40–80 mesh; flow-rate, 1 ml/min; column temperature, 22°. Derivative: azelaic acid–thiogalactose. Given values are from single or duplicate runs.

E.U. added	*Recovered*			
	Tris fraction (E.U.)	*Borate Fraction*		
		E.U.	*Specific activity*	*%*
335	23, 19	322, 321	149, 225	96, 96
671	148, 67	453, 315	232, 178	67, 47
1006	241, 277	381, 472	132, 155	38, 47
1340	497, 612	815, 687	232, 180	60, 51

TABLE VIII

AFFINITY CHROMATOGRAPHY OF β-GALACTOSIDASE

PAPTG = *p*-Aminophenyl-β-D-thiogalactopyranoside. All assays utilised *o*-nitrophenylgalactopyranoside as substrate.

Enzyme source	*Affinity ligand*	*Specific activity (μmoles·min⁻¹·mg⁻¹)*		*Reference*
		Before chromatography	*After chromatography*	
E. coli	PAPTG	—	320*	3
E. coli	PAPTG	45	450**	4
Jack Bean	galactolactone	12.6	600***	5
A. niger	PAPTG	60	120§	6
A. niger	PAPTG	102	240§§	This work

* 28°, 0.23 m*M* substrate, pH 7.0.
** 37°, 0.23 m*M* substrate, pH 7.0.
*** 37°, 10 m*M* substrate (*para* isomer), pH 3.5.
§ 37°, 5 m*M* substrate, pH 4.0 (value corrected to conditions of footnote §§).
§§ 60°, 8.3 m*M* substrate, pH 4.0.

as a 1-g column of 80–120 mesh glass). Factors such as the cost of the affinity ligand, availability of crude protein and the importance of purification need to be considered in selecting the optimum column and operating conditions.

The use of affinity chromatography for the purification of β-galactosidase has now been accomplished by several groups. The results obtained by these workers are summarized in Table VIII. An exact comparison is difficult due to differences in assay procedures. We have conducted the assay at several different conditions to allow a useful comparison to be made. Jack bean appears to be an inexpensive source of galactosidase of very high specific activity. Our results with respect to purification efficiency were similar to those of Woychek and Wondolowski. We both utilized a relatively pure protein contaminated with a large quantity (92%) of carbohydrate stabilizer.

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REFERENCES

1 P. Cuatrecasas and C. B. Anfinsen, *Methods Enzymol.*, 22 (1971) 345.
2 A. Tomino and K. Paigen, in D. Zipser and J. Beckwith (Editors), *The Lac Operon*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1970.
3 E. Steers, P. Cuatrecasas and H. B. Pollard, *J. Biol. Chem.*, 246 (1971) 196.
4 P. J. Robinson, P. Dunnill and M. D. Lilly, *Biochim. Biophys. Acta*, 285 (1972) 28.
5 J. H. Kanfer, G Petrovich and R. A. Mumford, *Anal. Biochem.*, 55 (1973) 301.
6 J. H. Woychik and M. V. Wondolowski, *Biochim. Biophys. Acta*, 289 (1972) 347.
7 M. Lynn, *Chim. Ind.*, 106 (1973) 601.
8 C. R. Lowe, M. J. Harvey, D. B. Craven, M. A. Kerfoot, M. E. Hollows and P. D. G. Dean, *Biochem. J.*, 133 (1973) 507.
9 B. H. J. Hofstee, *Biochem. Biophys. Res. Commun.*, 53 (1973) 1137.