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SEPHAROSE DERIVATIVES CONTAINING CITRIC ACID AS AFFINITY LIGAND

PURIFICATION OF FUMARASE

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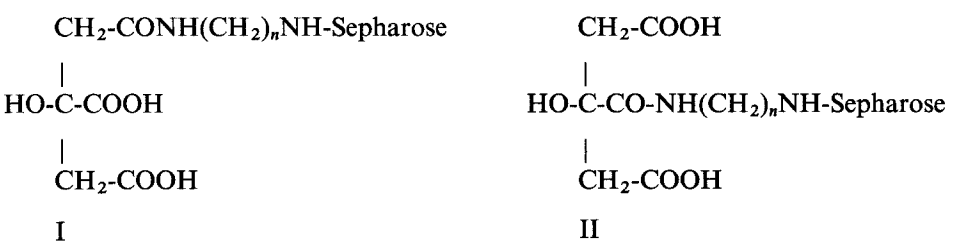
SUMMARY

Six Sepharose derivatives, in which citrate was immobilized via methylene carbons, were prepared by coupling of the α- and β-isomers of citrylpolymethylenediamine to Sepharose. The purification of fumarase from pig heart was dependent on the length of the spacer arm, but not on the isomeric configuration of the immobilized citrate. Gels having six methylene carbons had the largest adsorption capacity for the enzyme and therefore were the most suitable for use in affinity columns for its purification. Affinity chromatography with these gels was followed by hydrophobic interaction chromatography on an octamethylenediamine–Sepharose column.

INTRODUCTION

Enzyme purification by affinity chromatography is based on specific binding to a substance immobilized on a matrix of gels. Citrate, which is one of components of the tricarboxylic acid (TCA) cycle and is widely distributed in organisms, associates with several enzymes as a substrate or a regulator. A solid matrix containing immobilized citrate would be an affinity adsorbent that could be used for the purification of these enzymes.

In this study citric acid was bound through either the α- or β-carboxylic group of citrate and an amino group of polymethylenediamine (2, 4 or 6 methylene carbons) attached to Sepharose. Gels having three different polymethylene spacer arms, α- and β-CM_nD–Sepharose, I and II (n = number of polymethylene carbons), were prepared by the coupling of citrylpolymethylenediamine (CM_nD) with Sepharose activated by cyanogen bromide.



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These Sepharose derivatives were tested for the purification of fumarase (E.C. 4.2.1.2), of which citrate is an inhibitor. Affinity chromatography with these gels was followed by hydrophobic interaction chromatography with an octamethylenediamine (M_8D)–Sepharose column.

EXPERIMENTAL

Reagents and methods

NMR spectra were recorded with a JEOL FX-100 spectrometer. Compounds were dissolved in dilute NaO^2H) solution to obtain an aqueous solution of pH 7.0. Sodium 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate (TMSP) was used as an internal standard. In the special case of CM_2D the solvent was ^2H_2O acidified with a drop of 6 *M* hydrochloric acid, and TMSP was used as an external standard. Tricarballylic acid[1], and the α- and β-isomers of monomethyl citrate[2], were prepared as previously reported. All other chemicals were of commercially available reagent grade.

Thin-layer chromatography (TLC) was carried out with precoated plates of silica gel 60_{254} (E. Merck) and of cellulose (Avicel SF, Asahikasei Ind. Co.). The solvents were: A, ethanol–formic acid–water (18:1:1, v/v) for the silica plate; B, *n*-butanol–acetic acid–water (2:1:1, v/v) for the cellulose plate. Compounds were detected by spraying the chromatograms with a solution of 0.2% ninhydrin in acetone.

Preparation of α- and β-CM_nD

α- or β-monomethyl citrate (25 mmol) and polymethylenediamine (2, 4 or 6 methylene carbons; 125 mmol) were dissolved in 10 ml of water, and the resulting solution was stirred overnight at room temperature. The mixture was applied to a column (24 × 4 cm) of 300 ml of Amberlite IRA 900 (OH^-). The column was washed with water and then eluted with 0.5 *M* hydrochloric acid. The eluate was evaporated to dryness under reduced pressure at 40°C, and the residue was dissolved in a small volume of water. The solution was applied to a column (24 × 4 cm) of 300 ml of Diaion SK-1 (H^+). The column was washed with water and then eluted with 0.5 *M* ammonia. The eluate was evaporated to dryness under reduced pressure at 50°C, and the residue was dissolved with warming in the minimum volume of acetic acid. An equal volume of diethyl ether was added; the resulting precipitate was recrystallized from 70% ethanol in water, except for β-CM_6D which was recrystallized from water. Analytical data and yields (on the basis of monomethyl citrate) are as follows. α-CM_2D (monohydrate): yield 38%; m.p. 216–218°C; R_F 0.53 (solvent A) and 0.62 (solvent B); Calc. for $C_8H_{14}N_2O_6 \cdot H_2O$: C, 38.09; H, 6.32; N, 11.11%. Found: C, 37.97; H, 6.32; N, 10.88%. β-CM_2D: yield 27%; m.p. 177–179°C; R_F 0.38 (solvent A) and 0.53 (solvent B); Calc. for $C_8H_{14}N_2O_6$: C, 41.02; H, 6.02; N, 11.96%. Found: C, 40.50; H, 5.97; N, 11.57%. α-CM_4D: yield 39%; m.p. 189–191°C; R_F 0.18 (solvent A) and 0.57 (solvent B); Calc. for $C_{10}H_{18}N_2O_6$: C, 45.80; H, 6.92; N, 10.68%. Found: C, 45.38; H, 7.04; N, 10.55%. β-CM_4D: yield 26%; m.p. 181–182°C; R_F 0.42 (solvent A) and 0.61 (solvent B); Calc. for $C_{10}H_{18}N_2O_6$: C, 45.80; H, 6.92; N, 10.68%. Found: C, 45.37; H, 7.12; N, 10.40%. α-CM_6D (monohydrate): yield 30%; m.p. 146–148°C; R_F 0.35 (solvent A) and 0.62 (solvent B); Calc. for $C_{12}H_{22}N_2O_6 \cdot H_2O$: C, 46.75; H, 7.85; N, 9.08%. Found: C, 46.62; H, 7.47; N, 8.47%. β-CM_6D: yield 34%; m.p. 179–181°C; R_F 0.55 (solvent A) and 0.65 (solvent B); Calc. for $C_{12}H_{22}N_2O_6 \cdot H_2O$: C, 49.65; H, 7.64; N, 9.65%. Found: C, 49.34; H, 7.57; N, 9.51%.

Preparation of Sepharose derivatives

α- and β-CM_nD–, hexamethylene diamine (M_6D)–, octamethylenediamine (M_8D)– and ε-aminocaproic acid (ACA)–Sepharose. Wet Sepharose 4B (20 g) (Pharmacia, Uppsala, Sweden) was suspended in 20 ml of water and activated with cyanogen bromide (2 g)[3]. The activated gel was rapidly washed with cold water and then with 0.2 *M* $NaHCO_3$. The washed gel was immediately suspended in 40 ml of 0.2 *M* $NaHCO_3$ (pH 9.8) and 1.0 mmol of α- or β-CM_nD, or 5.0 mmol of M_6D, M_8D or ACA were added. The suspension was shaken overnight at 20°C, and then filtered. The gel was washed successively with 0.2 *M* $NaHCO_3$, water, 0.2 *M* sodium acetate buffer (pH 4.0) and water.

Fumarylhexamethylenediamine (FM_6D)– and tricarballylylhexamethylenediamine (TM_6D)–Sepharose. A 500-mg amount of fumaric acid or tricarballylic acid was added to a suspension of wet M_6D–Sepharose (15 g) in 30 ml of water. The pH of the suspension was adjusted to 5.0 by the addition of 1.0 *M* sodium hydroxide, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.0 g) in 2 ml of water was added in small portions. During the addition, the pH of the mixture was maintained at 4.8–5.0 by the addition of 1 *M* sodium hydroxide. The mixture was shaken overnight at room temperature. The gel was filtered off and washed as described above.

L-*Aspartic acid–Sepharose (ASP–Sepharose).* Wet Sepharose 4B (15 g) was activated with 1,4-butanediol diglycidyl ether (15 ml)[4]. The activated gel was washed extensively with water and suspended in 30 ml of 1 *M* $NaHCO_3$. After the addition of L-aspartic acid (0.5 g), the suspension was shaken for 12 h at 40°C. The gel was filtered off and washed as described above.

Determination of ligands bound to Sepharose

Ligands on CM_nD– and ACA–Sepharose were estimated by titration of the carboxylic groups on the gels with a standard sodium hydroxide solution[5]. The amino groups on M_6D– and M_8D–Sepharose were determined by a minor modification of the method reported[6]. The amino groups were reacted with 2,4,6-trinitobenzenesulphonate, and the resulting trinitrophenylated gel was dissolved by warming with a solution of 50% acetic acid in 3 *M* hydrochloric acid for 10 min at 50°C. The absorbance of the solution was measured at 340 nm, and the concentration of amino groups was calculated by use of the molar extinction coefficient, $\varepsilon = 1.49 \cdot 10^4$ l mol^{-1} cm^{-1}. The ligand concentrations of FM_6D– and TM_6D– Sepharose, which were prepared by coupling fumaric acid and tricarballylic acid to M_6D–Sepharose, were determined from the difference in the concentrations of amino groups on M_6D–Sepharose before and after the coupling reaction.

Assay of fumarase

Fumarase was assayed spectrophotometrically by following the production of fumarate from L-malate at 250 nm[7]. The number of activity units was expressed as 10^3 × (initial change in absorbance)/10 sec at 25°C in the reaction mixture (3 ml) placed in a cuvette (light path: 1 cm).

Protein determination

Protein concentrations were determined by use of bovine serum albumin as the standard[8].

Preparation of crude fumarase

Fresh pig hearts chilled in ice at a slaughterhouse were trimmed of fat and connective tissue and were immediately frozen at −20°C. Extraction of fumarase from the muscle and subsequent fractionation with ammonium sulphate were performed as described[7]. Crude fumarase (specific activity, 340–380 units/mg) was obtained from 200 g of muscle and was stored in 1.0 *M* ammonium sulphate at 4°C.

Chromatography on a CM_6D–Sepharose column

An aliquot of the above ammonium sulphate suspension (55,000 units) was centrifuged at 5000 *g*. The precipitate was dissolved in a solution (buffer A) of 10 m*M* Tris–acetate buffer (pH 7.3) containing 10 m*M* 2-mercaptoethanol (MSH). The solution was desalted on a Sephadex G-25 (fine) column (12 × 1 cm) equilibrated with buffer A and was applied to a column (8.0 × 0.9 cm) of CM_6D–Sepharose. The column was washed with buffer A until excess of fumarase was removed and then with a solution (buffer B) of 25 m*M* Tris–acetate buffer (pH 7.3) containing 10 m*M* MSH until the absorbance at 280 nm of the eluate was less than 0.03. Fumarase was eluted with a solution (buffer C) of 10 m*M* Tris–acetate buffer (pH 7.3) containing 1.0 m*M* fumarate, and fractions containing the enzyme activity were pooled for purification by chromatography.

Chromatography on a M_8D–Sepharose column

The pool of fumarase was applied to a M_8D–Sepharose column (13.0 × 1.0 cm) equilibrated with 20 m*M* sodium phosphate buffer (pH 7.3) containing 1.2 *M* ammonium sulphate and 10 m*M* MSH. A 30-ml linear decreasing gradient of 1.2–0.0 m*M* ammonium sulphate was used to elute the fumarase.

Inhibition experiments

Inhibition experiments with α- and β-CM_nD for fumarase were carried out measuring the initial rate of dehydration of L-malate in 50 m*M* Tris–acetate buffer (pH 7.3) at 25°C[9].

RESULTS

High resolution ^{13}C NMR spectra of CM_nD

The spectra of all the compounds α- and β-CM_nD revealed several sharp resonances. Their assignments are shown in Table I. The symmetrical molecule in the α-isomer series provided three resonances from the three different carbonyl carbons and two from the two different methylene carbons in the citric acid moiety. The symmetrical molecule in the β-isomer series showed two resonances from the identical carbonyl carbons in the carboxylic groups and carbonyl group of the amide group. In the case of CM_2D, two resonances from such carbonyl carbons were observed in an acidic solution. The two identical methylene carbons in the citric acid moiety yielded one resonance. In both series of isomers, the resonance due to the tertiary carbon atom in the citric acid moiety and to each of the methylene carbons in the polymethylenediamine moiety were separated from other resonances.

Ligand concentration of Sepharose derivatives

The ligand concentrations of the gels are listed in Table II. The concentrations were in the range of 5.4–9.8 μmol per ml of gel.

TABLE I

ASSIGNMENTS OF ^{13}C RESONANCES OF CM_nD

Samples were dissolved in dilute NaO^2H solution to obtain a solution of pH 7.0. Chemical shifts are in ppm downfield from TMSP.

	Chemical shift (ppm)			*Assignment*
	n=2	*n=4*	*n=6*	
α-Isomer	183.1	182.8	183.1	Carbonyl carbons
	181.5	181.2	181.5	
	176.2	175.2	175.1	
	77.9	77.6	77.8	Tertiary carbon
	48.4	48.2	48.0	Methylene carbons in citric acid residue
	47.6	47.2	47.2	
	41.9	41.6	42.2	Methylene carbons in polymethylene chain
	40.0	40.9	41.8	
		27.9	30.7	
		26.6	30.2	
			28.2	
			27.9	
β-Isomer	180.9 (179.1)*	180.9	180.8	Carbonyl carbons
	(176.1)	180.1	179.8	
	77.6 (76.7)	77.4	77.3	Tertiary carbon
	48.3 (45.9)	47.8	47.5	Methylene carbons in citric acid residue
	42.4 (42.1)	41.9	42.1	Methylene carbons in polymethylene chain
	40.4 (39.4)	41.0	41.6	
		28.1	30.8	
		27.0	29.9	
			28.0	
			26.7	

* Samples were dissolved in ^2H_2O and acidified with a drop of 6 *M* HCl.

Purification of fumarase

Chromatograms obtained on columns of α- and β-CM_6D–Sepharose were very similar, as shown in Fig. 1. Fumarase was retained on the columns equilibrated with 10 m*M* Tris–acetate buffer. Successive elution with increasing buffer concentrations up to 25 m*M* washed out other proteins. The retained enzyme was eluted by fu-

TABLE II

LIGAND CONCENTRATIONS OF SEPHAROSE DERIVATIVES

Sepharose derivative	*Concentration (μmol/ml of gel)*	*Sepharose derivative*	*Concentration (μmol/ml of gel)*
M_8D	6.8	α-CM_2D	7.0
ACA	9.8	β-CM_2D	7.6
FM_6D	5.4	α-CM_4D	8.1
TM_6D	5.6	β-CM_4D	7.6
ASP	5.4	α-CM_6D	7.9
		β-CM_6D	8.2

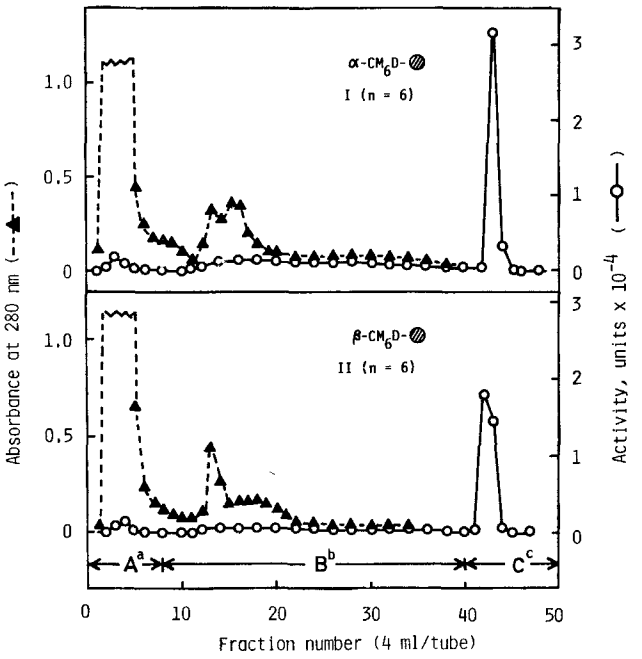

Fig. 1. Chromatograms of fumarase on α- and β-CM_6D–Sepharose columns. Crude fumarase (55,000 units) was applied to the column (8.0 × 0.9 cm), and the column was eluted successively with (a) 10 m*M* Tris–acetate buffer (pH 7.3) containing 10 m*M* MSH, (b) 25 m*M* Tris–acetate buffer (pH 7.3) containing 10 m*M* MSH and (c) 10 m*M* Tris–acetate buffer (pH 7.3) containing 1.0 m*M* fumarate. Fractions of 4.0 ml were collected at a flow-rate of 6.0 ml/h. ◍, Sepharose support.

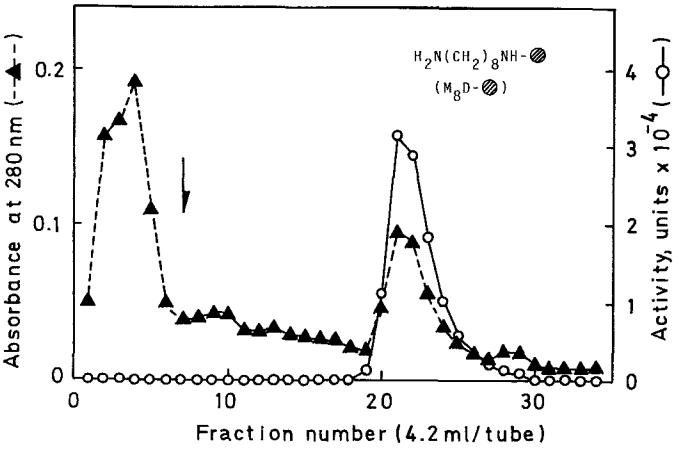

Fig. 2. Chromatogram on a M_8D–Sepharose column. Pooled fumarase (108,200 units) eluted from α-CM_6D–Sepharose was applied to a M_8D–Sepharose column (12.7 × 1.0 cm) equilibrated with 20 m*M* sodium phosphate buffer (pH 7.3) containing 1.2 *M* ammonium sulphate and 10 m*M* MSH. From the arrow a linear decreasing gradient was started: 1.2 *M* (100 ml) to 0.0 *M* ammonium sulphate (100 ml). Fractions of 4.2 ml were collected at a flow-rate of 8.4 ml/h. ◍, Sepharose support.

TABLE III

PURIFICATION OF FUMARASE FROM PIG HEART MUSCLE

Step		*Volume (ml)*	*Total protein (mg)*	*Specific activity*		*Recovery (%)*
				units	*units/mg*	
1	Crude extract*	215	5798	237,700	41	100
2	Ammonium sulphate fraction after desalting**	12	534	199,700	374	84
3	α-CM_6D–Sepharose	8	5	108,200	22,300	46
4	M_8D–Sepharose	30	3	103,300	35,700	43

* Obtained from 70 g of frozen heart muscle.
** Desalted with a Sephadex G-25 fine column.

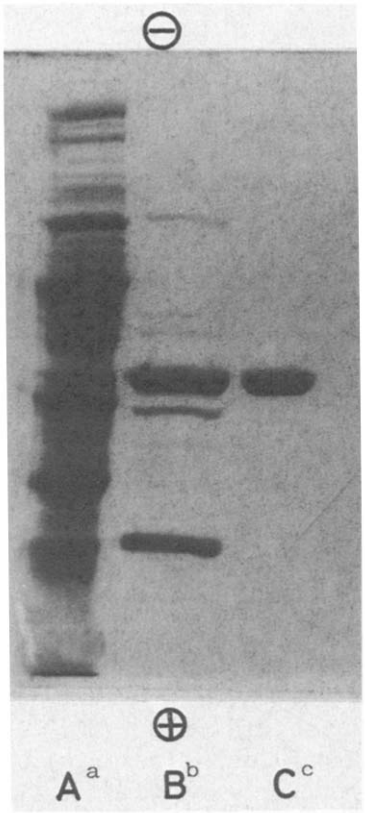

Fig.3. Polyacrylamide gel electrophoresis of fumarase. The electrophoresis was carried out in the system described by Laemmli[12]. The acrylamide concentration of the separating gel was 10%. a, A crude preparation; b, fractions eluted with fumarate from a α-CM_6D–Sepharose column; c, fraction eluted from a M_8D–Sepharose column.

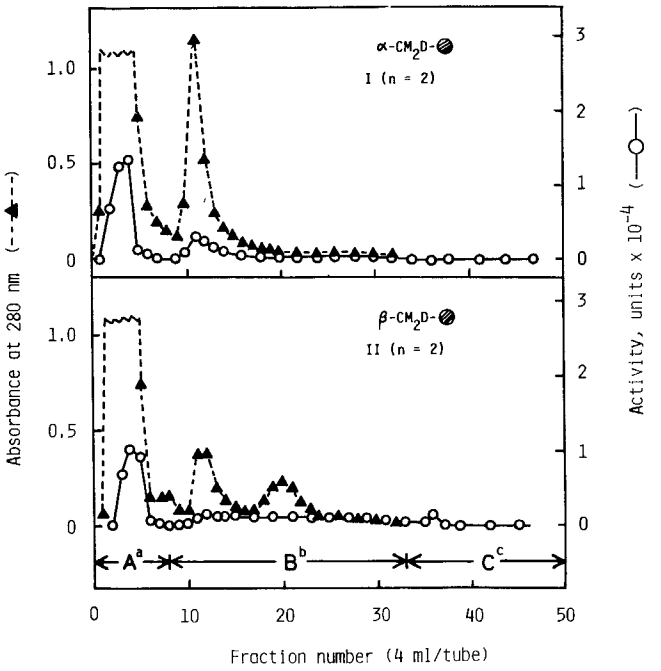

Fig. 4. Chromatograms on α- and β-CM_2D–Sepharose columns. Fumarase (54,000 units) was applied to each column. Conditions as in Fig. 1.

marate as a sharp fraction. Eluted fumarase was further purified on a M_8D–Sepharose column (Fig. 2). The enzyme was retained on the column with a high concentration (1.2 *M*) of ammonium sulphate, and was eluted with a linearly decreasing concentration gradient, 0.60–0.85 m*M*. A typical stepwise course of enzyme purification is outlined in Table III. Polyacrylamide gel electrophoresis of the crude extract and purified fractions obtained in the preparation are shown in Fig. 3.

Effect of length of spacer arm

Gels having two and four methylene carbons interposed between citrate and agarose were prepared, and the adsorption of fumarase was examined under chromatographic conditions as described for a CM_6D–Sepharose column. The chromatograms and specific activities of the enzyme fractions eluted by fumarate are shown in Figs. 4 and 5 and in Table IV, respectively. Elution with increasing buffer concentration (25 m*M*) of the gel with two methylene carbons ($n = 2$) washed out the bound enzyme, but not from the gel with four methylene carbons ($n = 4$). These findings indicate that firm binding of the enzyme can be obtained with a spacer arm of at least $n = 4$. The enzyme obtained from the gel with $n = 4$ showed almost the same specific activity as that obtained from gels with $n = 6$. However, these gels differed greatly in their adsorption capacities for the enzyme as seen from the recovery values. The gel with $n = 6$ had a much greater capacity than that with $n = 4$. No significant dependence of specific activity on capacity was observed.

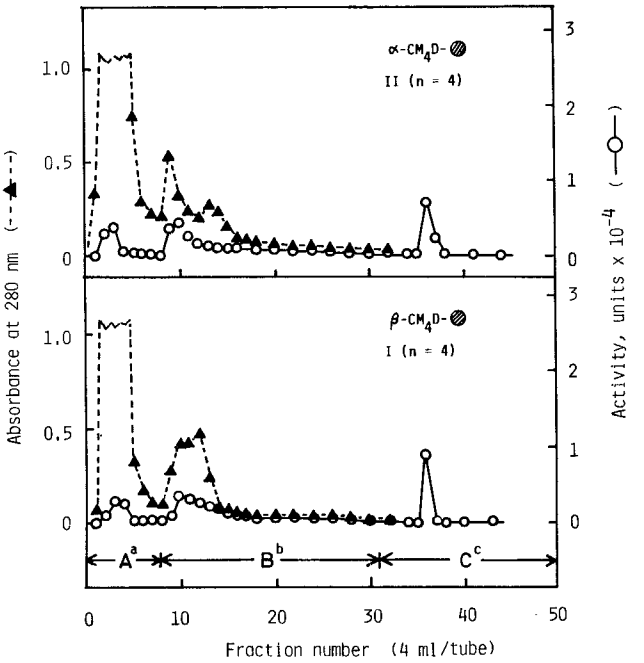

Fig. 5. Chromatograms on α- and β-CM_4D–Sepharose columns. Conditions as in Fig. 1.

Chromatography with other acidic Sepharose derivatives

Similar chromatographic experiments were carried out with four gels. The chromatograms are shown in Figs. 6 and 7. Gels of TM_6D–Sepharose retained the enzyme, which was eluted by fumarate with a low yield of 9%. Gels of FM_6D–, ASP– and ACA–Sepharose retained the enzyme, but the bound enzyme was eluted with increasing buffer concentration (25 m*M*).

Inhibition of fumarase with CM_nD

Inhibition profiles and inhibition constants are shown in Fig. 8 and Table V, respectively. All α- and β-isomers of CM_nD were found to be competitive inhibitors.

TABLE IV

PURIFICATION OF FUMARASE ON A CM_nD–SEPHAROSE COLUMN

n	*Isomer*	*Eluted fumarase*	
		Specific activity (units/mg)	*Yield (%)*
2	α	0	0
	β	0	0
4	α	23,200	17
	β	22,400	18
6	α	24,500	57
	β	26,000	62

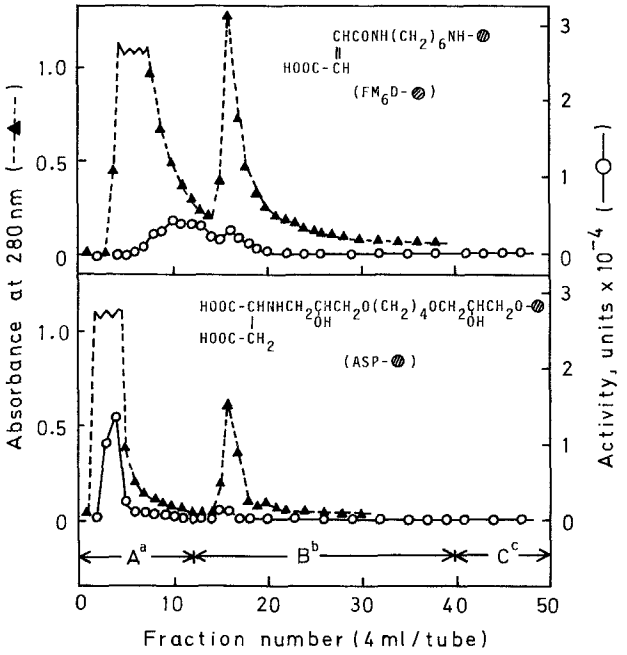

Fig. 6. Chromatograms on FM_6D- and ASP-Sepharose columns. Conditions as in Fig. 1. An exception is the absence of MSH in the eluent for a FM_6D-Sepharose column.

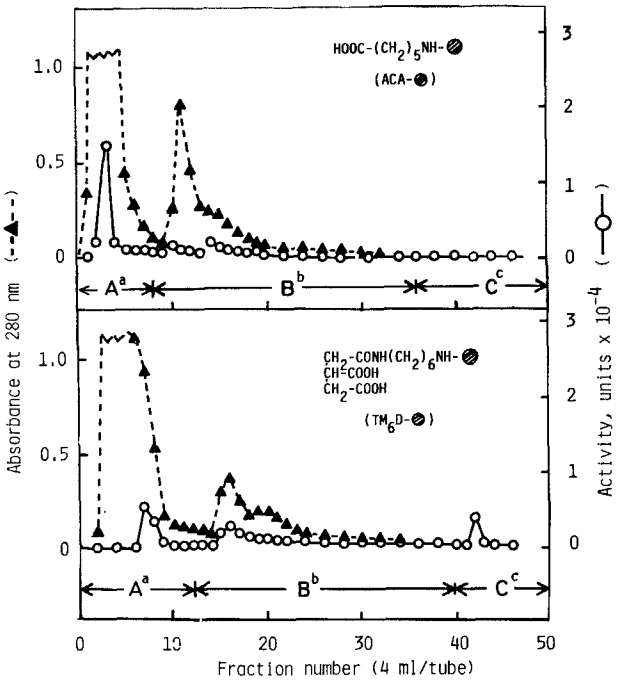

Fig. 7. Chromatograms on ACA- and TM_6D-Sepharose columns. Conditions as in Fig. 1.

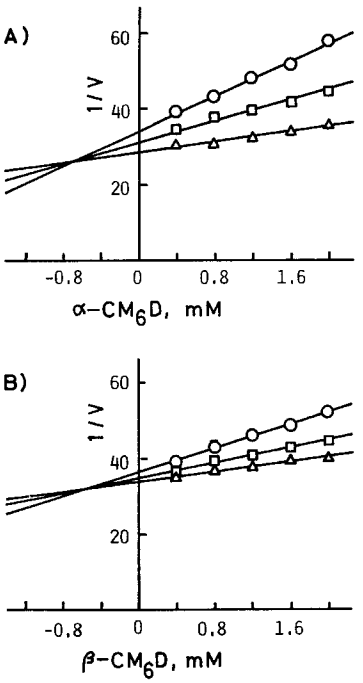

Fig. 8. Inhibitions on fumarase by α- and β-CM_nD. L-Malate concentrations were: ○, 1.6 m*M*; □, 8.0 m*M*; △, 40.0 m*M*. The initial rate, *V*, of production of fumarate was measured spectrophotometrically at 250 nm in 50 m*M* Tris–acetate buffer (pH 7.3 at 25°C). The competitive components of the inhibition were investigated by plotting $1/V$ against inhibitor concentration[9].

The inhibition constants K_i, of all the α-isomers were a little higher than those of all the β-isomers. The K_i values did not significantly change with the number of methylene carbons in the polymethylenediamine residue.

TABLE V

INHIBITION CONSTANTS OF FUMARASE WITH α- AND β-CM_nD

Isomer	*n*	K_i *(mM)*
α	2	0.70
	4	0.75
	6	0.70
β	2	0.50
	4	0.50
	6	0.50

DISCUSSION

The major part of this work was the preparation of α- and β-CM_nD for binding to Sepharose after reaction with cyanogen bromide. A simple preparation of asym-

metric citric anhydride, which we reacted with polymethylenediamine to produce an isomeric mixture of CM_nD, was reported previously[10]. However, we encountered difficulty with the separation of the two isomers produced in the reaction. Our successful method, which necessitated no separation of isomers, involved the aminolysis of α- and β-monomethyl citrate, which were easily prepared by partial esterification of citric acid, with polymethylenediamine. The identification and purity of the compounds obtained were confirmed by NMR spectroscopy, m.p. TLC, and elemental analysis.

The basic requirement for successful affinity chromatography is the formation of a specific complex of the enzyme with a ligand covalently bound to the solid support. At the beginning of this investigation we expected that a gel having either the α or β configurational isomer of citrate would form a more specific complex with fumarase than one having a random mixture of the isomers, and that one would serve as a more efficient affinity adsorbent than the other. However, both gels showed almost the same efficiency in the purification of the enzyme. This indicates that fumarase binds with the same specificity to either of the two isomers. The non-specificity might be responsible for a small difference in K_i values observed between fumarase and isomeric soluble ligands.

Several mechanisms, in principle, could be considered for the apparently specific binding of fumarase by the citrate-bound gels. Hydrophobic binding to the ligand spacer arm appears unlikely since the enzyme is efficiently eluted by a relatively low concentration of fumarate. Binding is therefore likely to occur either by a non-specific ion-exchange mechanism or by biospecific interaction of the citrate function of the bound ligand with fumarase. The fact that acidic ligands having carboxylic groups other than citric acid did not show firm binding is evidence in favour of a biospecific interaction. Soluble ligands behaved as competitive inhibitors for the substrate of the enzyme, as did citrate[11]. Therefore, the binding of the enzyme to immobilized citrate is likely to occur via the substrate binding site of the enzyme. The ligand tricarballylic acid, having a similar structure to citric acid, bound fumarase with other proteins. Such a low affinity might be due to the structure of tricarballylic acid, which lacks the hydroxy group present in citrate.The number of methylene carbons in soluble ligands did not affect their affinities as inhibitors of fumarase. However, when the ligand was immobilized on Sepharose, the number of methylene carbons influenced the binding power, adsorption capacity and purification factor of the enzyme. Four methylene carbons provided sufficient binding power and a satisfactory purification factor, but insufficient adsorption capacity. Therefore either the α- or the β-isomer of CM_6D–Sepharose is the most suitable adsorbent when adsorption capacity and purification factor are considered. Studies of citrate-bound Sepharose for the purification of other enzymes are in progress.

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