Single-Step Isolation and Resolution of Pancreatic Carboxypeptidases A and B†

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ABSTRACT: Carboxypeptidases A and B have been isolated individually from aqueous extracts of mammalian pancreatic acetone powders by affinity chromatography on [*N*-(ε-aminocaproyl)-*p*-aminobenzyl]succinyl-Sepharose 4B (CABS–Sepharose). The affinity ligand was synthesized from DL-benzylsuccinic acid, purified, and characterized by UV absorption and NMR spectroscopy. Both enzymes from the various species were homogeneous by $NaDodSO_4$–polyacrylamide gel electrophoresis and displayed high specific activities. No cross contamination of one enzyme species with the other was found. The ease of synthesis of the ligand from its commercially available precursor, its stability, and the mild elution conditions render CABS–Sepharose an excellent affinity support for the single-column isolation of both carboxypeptidases A and B. The procedures extend the utility of this resin previously demonstrated for carboxypeptidase A from human pancreatic juice [Peterson, L. M., Sokolovsky, M., & Vallee, B. L. (1976) *Biochemistry 15*, 2501]. The use of CABS–Sepharose as a general affinity matrix for the isolation of metallocarboxypeptidases is suggested.

The isolation and purification of pancreatic carboxypeptidases has generally required multiple fractionation procedures such as salt and solvent precipitation, gel permeation and ion-exchange chromatography, and recrystallization. Such laborious, time-consuming, and costly techniques together with low yields have precluded extensive comparative and evolutionary studies of this important class of proteolytic enzymes. Moreover, physicochemical differences between these enzymes as well as their contaminants in different species have necessitated the employment of specific experimental conditions in each particular instance.

During the last decade, affinity chromatography has greatly facilitated the isolation of enzymes, homogeneous by physicochemical criteria. However, the successful application of this principle of isolation to the carboxypeptidases lagged owing to nonspecific adsorption (Uren, 1971; Reeck et al., 1971), the need for chaotropic elution conditions (Cuatrecasas, 1968), or lengthy procedures for isolation of sufficient quantities of ligand of adequate chemical stability (Ryan et al., 1974).

An affinity adsorbent consisting of the *p*-amino derivative of DL-benzylsuccinic acid,[1] a potent competitive inhibitor of carboxypeptidase (Byers & Wolfenden, 1972), coupled to caproyl-Sepharose has been employed recently for the isolation of carboxypeptidase A from human pancreatic juice (Peterson et al., 1976). The present study details the synthesis and characterization of this new affinity adsorbent and its application to the isolation of carboxypeptidases from activated extracts of mammalian pancreatic acetone powders from several species. The results indicate that carboxypeptidases A and B can be obtained in high purity and excellent yield by chromatography of such extracts on a single affinity column. The low cost and ready availability of the affinity ligand, its chemical stability, and the ideal binding characteristics of the immobilized inhibitor render this affinity resin a versatile tool for the rapid isolation of enzymes exhibiting carboxypeptidase specificity from a variety of sources.

Materials and Methods

Bovine carboxypeptidase A (Anson), porcine carboxypeptidase B, bovine trypsin, and α-chymotrypsin were obtained from Worthington Biochemical Corp. Bovine carboxypeptidase A (Cox) was from Sigma Chemical Co. Procarboxypeptidase A-S5 was prepared according to the method of Uren & Neurath (1972). Pancreatic acetone powders were purchased from Roth Products, Inc., and Pel-Freez Biologicals, Inc. Z-Gly-L-Phe, Bz-Gly-L-Arg, arginine, TosArgOMe, AcTyrOEt, and DCC were from Sigma Chemical Co. DL-Benzylsuccinic acid was obtained from Burdick and Jackson Laboratories, Inc., ε-aminocaproic acid was from Cyclo Chemical Corp., and Sepharose 4B was from Pharmacia Fine Chemicals.

NMR and UV absorption spectra were recorded with Varian A-60 and Cary 14 instruments, respectively. A_{280} of column effluents was measured with a Gilson Holochrome absorbance monitor. Salt gradients were prepared with a Pharmacia gradient mixing chamber.

$NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Weber & Osborn (1969) with the modifications described by Reeck et al. (1971). Commercial proteins were used as molecular weight markers.

Carboxypeptidase concentrations were estimated by using $A_{280}^{0.1\%} = 2.0$ when literature values were unavailable.

Zinc was determined by atomic absorption spectroscopy (Fuwa & Vallee, 1963). For ovine and equine carboxypeptidases, metal stoichiometries were based on an assumed molecular weight of 34 500.

Activity Measurements. Carboxypeptidase A activity was determined by using 20 mM Z-Gly-Phe in 1.0 M NaCl and 50 mM Tris, pH 7.5 (Auld & Vallee, 1970). Carboxypeptidase B activity was assayed by the same procedure with 1 mM Bz-Gly-Arg in 0.1 M NaCl and 50 mM Tris, pH 7.5.

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[1] Abbreviations used: DL-benzylsuccinic acid, 2-benzyl-3-carboxypropionic acid; TosArgOMe, N^{α}-(*p*-toluenesulfonyl)-L-arginine methyl ester; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CABS, [*N*-(ε-aminocaproyl)-*p*-aminobenzyl]succinyl-Sepharose 4B; Z-Gly-Phe, *N*-carbobenzoxyglycyl-L-phenylalanine; Bz-Gly-Arg, *N*-benzoylglycyl-L-arginine; DCC, *N*,*N*′-dicyclohexylcarbodiimide; $NaDodSO_4$, sodium dodecyl sulfate.

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Trypsin and chymotrypsin activities were measured potentiometrically with TosArgOMe and AcTyrOEt as the respective substrates (Walsh, 1970; Wilcox, 1970).

Preparation of Pancreatic Extracts. Pancreatic extracts were prepared by suspension of pancreatic acetone powder in water (10 mL/g of powder) with stirring overnight at 4 °C. The mixture was then centrifuged at 12000*g* for 20 min, and the supernatant was filtered through glass wool. The pH was adjusted to 8.0 with 1 M NaOH and trypsin was added (1 mg/g of acetone powder). After incubation for 2 h at 25 °C, starting conditions for affinity chromatography were established by adding concentrated NaCl solution and solid Mes.

Synthesis of DL-(*p-Aminobenzyl*)*succinic Acid.* (*a*) DL-(*p-Nitrobenzyl*)*succinic Acid.* DL-(*p*-Nitrobenzyl)succinic acid was prepared by dropwise addition over a period of 4 h of 0.05 mol of nitric acid to 10.3 g (0.05 mol) of DL-benzylsuccinic acid which was dissolved in 20 mL of concentrated sulfuric acid. The temperature was kept below 0 °C throughout the reaction. The mixture was then poured over crushed ice and immediately extracted with ethyl acetate. The ethyl acetate layer was washed 3 times with a saturated NaCl solution and dried over anhydrous magnesium sulfate, and the volume was reduced to 5 mL on a rotary evaporator. Upon addition of 500 mL of chloroform, tan crystals formed within 2–3 days at 4 °C. The crude product was dissolved in boiling water and decolorized with activated charcoal, and crystallization was allowed to proceed at 4 °C. The crystals were collected in a sintered glass funnel and dried in vacuo over phosphorus pentoxide. Yield, 64%; mp 149–151 °C uncor; NMR (Me_2SO-d_6) δ 2.42 (m, 2H), 3.0 (m, 3H), 7.9 (A_2B_2, 4H); UV (EtOH) 272 nm (ϵ 1.3 × 10^4 M^{-1} cm^{-1}).

(*b*) DL-(*p-Aminobenzyl*)*succinic Acid.* DL-(*p*-Nitrobenzyl)succinic acid was reduced by catalytic hydrogenation. Five grams (0.02 mol) of (*p*-nitrobenzyl)succinic acid was dissolved in 50 mL of methanol and treated with 0.5 g of 1% Pd–charcoal and hydrogen at atmospheric pressure with vigorous stirring; hydrogen uptake 1.31 L (97% of theory). The reaction mixture was filtered and evaporated at reduced pressure to a thick syrup. The residue was taken up in 20 mL of boiling water, decolorized with charcoal, and filtered. Colorless crystals formed overnight at 4 °C. Yield, 75%; mp 189–190 °C; NMR (Me_2SO-d_6) δ 2.4(m, 2H), 2.7 (m, 3H), 6.75 (A_2B_2, 4H); UV (EtOH) 239 nm (ϵ 9.9 × 10^3 M^{-1} cm^{-1}).

Preparation and Capacity of CABS-Sepharose. ϵ-Aminocaproic acid was coupled to Sepharose 4B by the cyanogen bromide procedure (Cuatrecasas & Anfinsen, 1971). DL-(*p*-Aminobenzyl)succinic acid was conjugated with caproyl-Sepharose via the *N*-hydroxysuccinimide ester (Cuatrecasas & Parikh, 1972). A 50-mL portion of packed caproyl-Sepharose was washed with anhydrous dioxane, suspended in 75 mL of dioxane, and stirred for 90 min with 1.6 g of *N*-hydroxysuccinimide and 3.0 g of DCC. The activated resin was washed extensively with dioxane, methanol and again dioxane, filter-dried, and transferred to an ice-cold solution of 1.6 g of DL-(*p*-aminobenzyl)succinic acid in 150 mL of sodium phosphate, pH 7.0. After stirring overnight at 4 °C, the resin was washed with 0.2 M sodium bicarbonate, pH 8.8, stirred for 6 h in 0.2 M glycine at pH 8.8, washed with 1 M NaCl, and finally washed with 20 mM Mes, pH 6.0. The resin was stored at 4 °C in the presence of sodium azide. The adsorbent retained specificity and practically unaltered binding capacity for numerous experiments in the course of more than 1 year.

Binding capacity of CABS–Sepharose for carboxypeptidase was determined by stirring a measured amount of resin with a solution containing excess carboxypeptidase at the desired pH and ionic strength at 23 °C. After centrifugation, the amount of protein adsorbed was calculated from the difference in absorbance at 278 nm of the supernatant (ϵ_{278} 6.42 × 10^4 M^{-1} cm^{-1}; Simpson et al., 1963) before and after equilibration with CABS–Sepharose.

Affinity Chromatography. Samples were applied to a 0.9 × 10 cm CABS-Sepharose column equilibrated with 0.5 NaCl and 20 mM Mes, pH 6.0, at 23 °C. The column was washed with equilibration buffer until A_{280} of the effluent indicated removal of nonbinding proteins. Bound proteins were then desorbed either by adding free, competitive ligand to the eluent or by increasing the eluent pH and ionic strength as specified in Figures 1–3.

Table I: pH and Ionic Strength Dependence of the Binding Capacity of CABS-Sepharose for Carboxypeptidase A

	binding capacity (mg/mL)			
pH	0 NaCl	0.2 M NaCl	0.5 M NaCl	1.0 M NaCl
6	14.2	6.6	3.6	2.0
7	6.6	1.7	0.7	0.6
8	3.3	0.4	0.1	<0.1

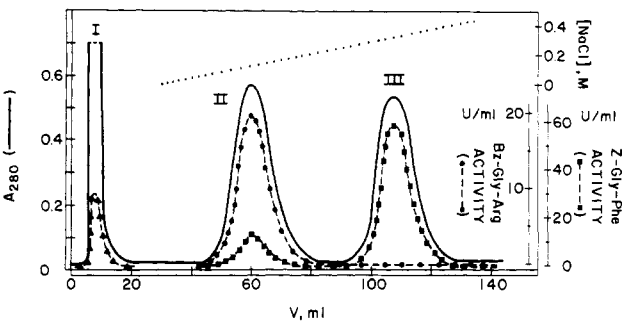

FIGURE 1: Resolution of proteins on CABS–Sepharose. A mixture of bovine serum albumin, bovine γ-globulin, horse hemoglobin, bovine carboxypeptidase A, and porcine carboxypeptidase B, 4 mg each, was applied to the column in 2 mL of equilibration buffer (20 mM Mes buffer, pH 6.0, and 0.5 M NaCl). The column was then washed with 20 mL of equilibration buffer and 10 mL of Mes buffer, pH 6.0, in the absence of salt to elute nonbinding proteins (peak I). Carboxypeptidase B (peak II) and carboxypeptidase A (peak III) were desorbed in a linear NaCl gradient (0–0.5 M, 120 mL) at pH 7.5. Resin, 10 × 0.9 cm CABS-Sepharose; flow rate, 0.8 mL/min; *T*, 23 °C. (—) A_{280}; (●) Bz-Gly-Arg hydrolysis; (■) Z-Gly-Phe hydrolysis; (···) NaCl gradient. Procarboxypeptidase A [A_{280} (▲)] was applied to the column in a separate experiment.

Results

Binding of bovine carboxypeptidase A to CABS–Sepharose strongly depends on the pH and salt concentration (Table I). At pH 6.0, in the absence of NaCl, 14.2 mg of carboxypeptidase binds to 1 mL of resin. Increasing the salt concentration to 1 M NaCl at constant pH decreases the binding capacity to 2 mg/mL. The sodium chloride concentration is even more critical to enzyme binding at higher pH: adsorption is virtually abolished at pH 8.0 in the presence of 0.5 M NaCl. Thus, within a pH and salt concentration range which does not affect carboxypeptidase activity adversely, enzyme binding to the affinity resin can be varied greatly. A qualitatively similar adsorption pattern was obtained for porcine carboxypeptidase B. No significant adsorption of carboxypeptidase A or B to unliganded caproyl-Sepharose was observed, even at pH 6 and low ionic strength. Both carboxypeptidase A and B can, therefore, be separated quantitatively from a mixture with other proteins (Figure 1). When applied at pH 6.0 in

Table II: Isolation of Carboxypeptidase A and B from Aqueous Extracts of Bovine Pancreatic Acetone Powder by Affinity Chromatography on CABS-Sepharose

	protein (mg)	Bz-Gly-Arg act. (units[a]/mg)	Z-Gly-Phe act. (units/mg)	Zn (g-atom/mol)	yield (%)
activated extract	251	251	0.8		100
fraction I (nonbinding)	235	0.2	3.5		
fraction II (elution with L-arginine)	2.0	71	39	0.96	74
fraction III (elution with NaCl gradient)	3.9	0	202	0.98	48

[a] One unit = 1 μmol of substrate hydrolyzed per min.

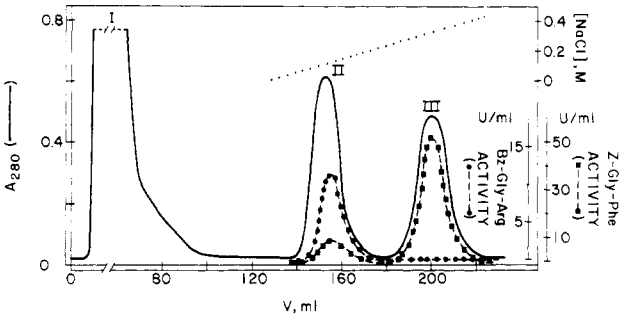

FIGURE 2: CABS–Sepharose chromatography of bovine pancreatic proteins. Activated aqueous extract (35 mL) of bovine pancreatic acetone powder in 0.5 M NaCl and 20 mM Mes, pH 6.0, was applied to the CABS–Sepharose column. The column was subsequently washed with 40 mL of equilibration buffer and 40 mL of pH 6 Mes buffer to elute nonbinding proteins (peak I). Adsorbed proteins (peaks II and III) were then eluted at pH 7.5 with a linear salt gradient. Experimental conditions and symbols are as shown in Figure 1.

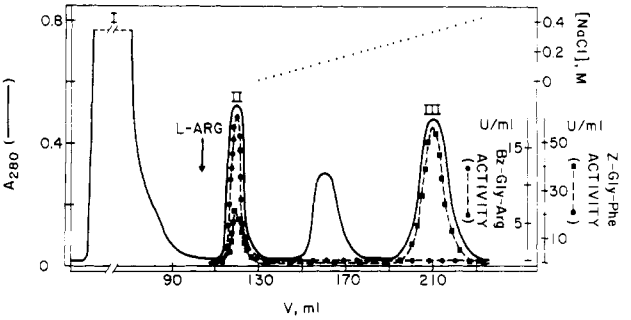

FIGURE 3: Isolation of pure carboxypeptidases A and B by single-column affinity chromatography on CABS–Sepharose. The activated extract (40 mL) of bovine pancreatic acetone powder was applied to a 0.9 × 10 cm column. Nonbinding proteins (peak I) were eluted at pH 6.0 with 40 mL of 0.5 M NaCl and 40 mL of salt-free Mes buffer. Carboxypeptidase B (peak II) was desorbed with 10 mM L-arginine in 20 mM Mes, pH 6.0. Proteins devoid of Z-Gly-Phe activity, followed by carboxypeptidase A (peak III), were eluted at pH 7.5 with a 0–0.5 M linear NaCl gradient.

0.5 M NaCl to minimize protein adsorption due to nonspecific ionic interaction, proteins devoid of carboxypeptidase activity elute unretarded from the affinity column (peak I) while carboxypeptidases B (peak II) and A (peak III) adhere to the resin. As expected from their virtually identical binding to the soluble inhibitor (Byers & Wolfenden, 1973; Zisapel & Sokolovsky, 1974), they are both desorbed in similar fashion by increasing the pH and salt concentration. Resolution is achieved at pH 7.5 through a sodium chloride concentration gradient. Quantitative recovery, specific activities, and polyacrylamide gel electrophoresis indicate complete separation of carboxypeptidases A and B from accompanying protein and excellent resolution of the two enzymes.

In a separate experiment, procarboxypeptidase A was applied to the CABS-Sepharose column (Figure 1). In spite of its intrinsic carboxypeptidase activity (Uren & Neurath, 1974; Bazzone & Vallee, 1976) which is inhibited by soluble benzylsuccinic acid (Bazzone, 1974), the zymogen was not retarded on the column.

CABS–Sepharose Chromatography of Pancreatic Extracts. By use of the same procedure, a 40-mL aliquot of aqueous extract of activated bovine pancreatic acetone powder was passed over CABS–Sepharose (Figure 2). More than 90% of the total amount of protein applied eluted on sample loading and washing the column with the equilibration buffer (0.5 M NaCl and 20 mM Mes, pH 6.0; peak I). Besides chymotrypsin and trypsin activity, approximately 50% of carboxypeptidase A and 25% of carboxypeptidase B activity were detected in peak I. Upon elution at pH 7.5 with a salt gradient, 74% of the total carboxypeptidase B activity applied emerged in a protein peak fraction (peak II) centered around 0.1 M NaCl. Low specific activity toward Bz-Gly-Arg, a low Zn to protein ratio, and rapid hydrolysis of AcTyrOEt and TosArgOMe indicated contamination of this fraction with chymotrypsin and trypsin (Table II). The specific activity of peak III toward Z-Gly-Phe was maximal near 0.35 M NaCl, characteristic of pure carboxypeptidase A; absence of Bz-Gly-Arg activity, a zinc to protein ratio of 0.97 g-atom/mol, and a single protein band (molecular weight 34 000) in polyacrylamide gels containing $NaDodSO_4$ indicated that this peak consists of essentially pure carboxypeptidase A.

Table III: Purification of Carboxypeptidase A from Aqueous Extracts of Pancreatic Acetone Powders

species	units applied	sp act. in extract (units/mg)[a]	sp act. of pure enzyme[b] (units/mg)[a]	Zn (g-at cm/mol)	yield (%)
cow	1659	6.6	202	0.98	48
horse	2715	2.6	208	1.10	84
sheep	1131	5.8	186	0.84	65

[a] Activity of commercial bovine enzyme (Worthington): 180–190 units/mg. [b] Pooled peak III as shown in Figure 2.

Simultaneous elution of carboxypeptidase B with chymotrypsin and trypsin was circumvented by specific desorption of carboxypeptidase B using a soluble competitive ligand (Figure 3). Nonbinding protein (peak I) was first eluted by washing with 0.5 M NaCl at pH 6.0, followed by a pH 6 buffer without salt. Addition of L-arginine (10 mM) to the eluent leads to the emergence of a sharp protein peak within 3 column volumes. After dialysis against metal-free buffer (0.1 M NaCl and 50 mM Tris, pH 7.5), this fraction exhibited high specific activity toward Bz-Gly-Arg, no activity toward AcTyrOEt and TosArgOMe, and 0.96 g-atom of zinc per mol of protein and was homogeneous by electrophoresis. Thus, this fraction is carboxypeptidase B of high purity (Table III). On subsequent elution with a linear NaCl gradient at pH 7.5, a peak of protein devoid of Z-Gly-Phe activity but displaying chymotrypsin and trypsin activity emerged from the column, followed by carboxypeptidase A (peak III). Analogous

chromatographic profiles and activity patterns were obtained with extracts of acetone powders of equine and ovine pancreatic tissue (Table III). In each instance washing with 0.1 M L-arginine at pH 6.0 displaces carboxypeptidase B from the affinity column. Carboxypeptidase A was then desorbed with a linear sodium chloride gradient at pH 7.5. Purity was confirmed by measurements of activity, zinc content, and gel electrophoresis.

Discussion

Although a number of carboxypeptidase affinity resins have been reported, none have proven suitable for the simultaneous isolation and resolution of carboxypeptidases A and B. L-Tyr-D-Trp-Sepharose selectively binds carboxypeptidase A at pH 8 (Cuatrecasas et al., 1968). However, desorption of the enzyme from this resin requires denaturing conditions. Substitution of ϵ-aminocaproic acid for L-Tyr as a spacer molecule in this resin resolves this problem (Reeck et al., 1971), but simultaneous adsorption of carboxypeptidase B, chymotrypsin, and trypsin occurs when pancreatic extracts are applied to the resin. Similar problems were encountered with insolubilized glycyl-D-phenylalanine (Uren, 1971). An affinity procedure employing a polypeptide inhibitor of carboxypeptidase, isolated from potatoes, has recently resulted in greatly improved resolving power (Ager & Hass, 1977). However, in that instance, while no interference from adsorption of other proteases was encountered, carboxypeptidases A and B were bound so tightly that they could not be dissociated from the column differentially. Dissociation and separation of the A and B enzymes were achieved by means of a pH 11.4 buffer, followed by a second chromatographic step on immobilized D-arginine.

Benzylsuccinic acid is available commercially, and coupling of the readily synthesized *p*-amino derivative to caproyl-Sepharose results in a resin with excellent chemical stability and resistance to proteolytic degradation. The adsorbed enzymes are eluted differentially in one step under mild conditions which do not alter protein conformation.

Salt dependence of adherence to an affinity ligand is generally thought to reflect ion-exchange effects (O'Carra et al., 1974). The decreasing binding capacity for carboxypeptidase with increasing sodium chloride concentration observed with CABS-Sepharose might therefore indicate nonbiospecific adsorption. However, decreasing pH increases the specific binding capacity to the resin, contrary to what would be expected if the dicarboxylic acid ligand were acting as a general anion exchanger and consistent with the inhibiting effects of the ligand in solution. Further, carboxypeptidase does not adhere to a "mock" resin consisting of the spacer molecule ϵ-aminocaproic acid immobilized on Sepharose 4B.

Desorption of carboxypeptidase B with L-arginine (Figure 3) further corroborates the biospecific affinity nature of the enzyme–CABS–Sepharose interaction. If binding of the immobilized ligand to the active center is the predominant force responsible for retention of the protein on the matrix, addition of a soluble active-site-directed ligand should compete for binding to the protein and promote elution (Dunn & Chaiken, 1974). L-Arginine competitively inhibits carboxypeptidase B but not carboxypeptidase A (Wolff et al., 1962). Thus, a 10 mM solution of L-arginine in Mes buffer at pH 6.0 elutes only carboxypeptidase B, while a 0.1 mM solution of benzylsuccinic acid liberates both carboxypeptidase A and carboxypeptidase B (L. Cueni, unpublished experiments). Binding of enzyme to CABS–Sepharose is, therefore, thought to occur at the active center in a mode analogous to that which prevails for the soluble inhibitor.

Table III illustrates the general applicability of CABS–Sepharose for the isolation and preparation of carboxypeptidase A. The high degree of active site-directed selectivity of DL-benzylsuccinic acid accounts for the uniform results obtained with carboxypeptidases from different species (Byers & Wolfenden, 1973). This selectivity appears to extend to carboxypeptidase B as well since patterns for the ovine and equine pancreatic extracts are very similar to that for the bovine extract (Figure 2).

Procarboxypeptidase A exhibits intrinsic enzymatic activity toward typical substrates of carboxypeptidase A (Uren & Neurath, 1974; Bazzone & Vallee, 1976). However, kinetics of substrate hydrolysis have revealed distinct differences in the active-site binding characteristics of the zymogen compared with those of the enzyme. The change in protein structure, brought about by zymogen activation, is reflected in the apparent lack of zymogen binding to the immobilized inhibitor under the conditions employed. Since the intrinsic activity of the zymogen is inhibited by DL-benzylsuccinic acid, it seems likely that proper solvent conditions and/or a change in the mode of attachment of the inhibitor to the resin might well provide a means for its selective adsorption.

The passage of a substantial amount of bovine carboxypeptidase A compared to the equine and ovine enzyme on chromatography of pancreatic extracts is attributed to this capacity of CABS–Sepharose to discern between the activated enzyme and its precursor (Figure 1). Full activation of bovine carboxypeptidase A is known to require exposure to a relatively high concentration of trypsin and/or prolonged incubation (Yamasaki et al., 1963). Since no attempt was made to optimize activation, a significant fraction of enzymatically active proenzyme could be present in the initial bovine pancreatic extract.

The data demonstrate that chromatography on CABS–Sepharose permits the single-step isolation of highly purified carboxypeptidase A or B from extracts of pancreatic powders from different species. Combination of buffer gradient elution and biospecific desorption with a free competitive ligand allows isolation of both carboxypeptidase A and carboxypeptidase B as separate entities. Refinement of buffer elution procedures should further simplify these methods.

Assuming active-site homology, the same affinity matrix might serve for the isolation of nonmammalian carboxypeptidases. Consistent with this expectation, the bacterial zinc carboxypeptidase from *Streptomyces griseus* has recently been shown to bind to CABS-Sepharose (Breddam et al., 1979).[2] This resin may, therefore, serve as a general affinity adsorbent for the isolation and purification of metallocarboxypeptidases in lower forms of life and, thus, facilitate comparative genetic studies of this important class of enzymes.

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Rapid Preparation of Mitochondrial Malate Dehydrogenase from Rat Liver and Heart†

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ABSTRACT: Mitochondrial malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) has been purified from both rat liver and rat heart by using Sepharose–Blue Dextran pseudoaffinity chromatography. Under the conditions employed most of the mitochondrial malate dehydrogenase and some cytosolic malate dehydrogenase are adsorbed, whereas many proteins simply pass through. The blue column is then subjected to several treatments which elute a variety of other proteins (notably residual cytosolic malate dehydrogenase and lactate dehydrogenase) which also have an affinity for the bound dye Cibacron Blue F3GA. Mitochondrial malate dehydrogenase is then eluted by forming an abortive ternary complex with reduced nicotinamide adenine dinucleotide–sodium D(+)-malate. Pooled active fractions are passed through a preequilibrated diethylaminoethyl–Sephadex column which retains contaminating proteins. The active fractions are concentrated, and residual contaminants are removed by very small stepwise pH changes on a CM-52 cellulose column. The procedure is mild and rapid and yields enzyme which is homogeneous by the applied criteria. In addition, lactate dehydrogenase may be copurified. The purified mitochondrial malate dehydrogenases from liver and heart have been characterized and compared. Both enzymes show evidence of multiple forms upon starch or polyacrylamide gel electrophoresis or upon isoelectric focusing. These forms are not generated artifactually during purification since they correspond to forms seen in crude extracts. The heart enzyme contains more high p*I* forms than the liver enzyme. Sodium dodecyl sulfate electrophoresis indicated identical subunit molecular weights of 35 000 for enzyme from both sources. Ultraviolet spectra were practically identical. The mild isolation procedure produces mitochondrial malate dehydrogenase of high (if not maximal) specific catalytic activity. Amino acid analyses of heart and liver mitochondrial malate dehydrogenase showed almost identical values, and the high levels of amide nitrogen are consistent with the high p*I* values (9.0–9.5) obtained by column electrofocusing. In addition, the procedure described produces enzymes with small, somewhat variable amounts of tightly complexed glycerophospholipids.

Mitochondrial malate dehydrogenase (m-MDH)[1] has been isolated and partially characterized from various sources (Ochoa, 1955; Wolfe & Neilands, 1956; Davies & Kun, 1957; Thorne, 1960; Grimm & Doherty, 1961; Sophianopoulos & Vestling, 1962; Fahien & Strmecki, 1969; Glatthaar et al., 1974). The isolation procedures have been time consuming and the yields rather low. In the case of rat liver m-MDH, difficulties have been encountered with respect to the stability of the purified enzyme and with respect to limited proteolysis and/or deamidation which led to the apparent modification of one of the two subunits but to no loss of maximum specific activity (Mann & Vestling, 1968, 1969, 1970).

We have developed an efficient rapid isolation procedure for m-MDH using Sepharose–Blue Dextran affinity chromatography (Ryan & Vestling, 1974). We have also reexamined our evidence for the existence of nonidentical subunits in the case of rat liver m-MDH. If care is taken to avoid limited proteolysis and/or deamidation, only one subunit is produced in the procedure to be described. This result is noted under subunit dissociating conditions during polyacrylamide

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[1] Abbreviations used: m-MDH, mitochondrial malate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; CM, carboxymethyl; U, international MDH units; $NaDodSO_4$, sodium dodecyl sulfate.

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