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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS*

SHUNG HO CHANG
Sherwood Medical Industries, Inc., St. Louis, Mo. (U.S.A.)
and
KAREN M. GOODING and FRED E. REGNIER
Department of Biochemistry, Purdue University, West Lafayette, Ind. 47907 (U.S.A.)

SUMMARY

Proteins were separated on microparticulate bonded phase steric exclusion and anion-exchange chromatography supports. A post-column enzyme detector was developed which gives a specific and sensitive response for enzymes. The three isoenzymes of creatine phosphokinase were separated and assayed in 4 min and the five isoenzymes of lactic dehydrogenase in 6 min.

INTRODUCTION

Chromatographic resolution of proteins in clinical and research laboratories has been a lengthy process. This is due primarily to the carbohydrate and polyacrylamide gel materials used as column supports. Polar functional groups in these gel supports cause them to imbibe water and swell to many times their dry volume. Subsequent changes in pH, ionic strength, and solvent composition during column elution result in changes in column support volumes with concomitant changes in flow-rate and column efficiency. As a result, recycling ion-exchange supports causes sufficient bed compression after several gradient elutions making it necessary to repack the column. The use of forced flow and pressure also causes the support to compress. The unsuitability of conventional protein-resolving chromatographic support materials for high-performance chromatography is obvious.

It has recently been established that macroporous inorganic supports coated with a covalently bonded glycerylpropylsilyl layer may be used in the high-performance liquid chromatography (HPLC) of proteins, polynucleotides, and polysaccharides[1]. The organic layer derivatizes surface silanols while imbibing a layer of water and preventing sensitive biological macromolecules from making contact with the inorganic surface. This treatment decreases the adsorption and/or denaturation of sensitive biological compounds by controlled porosity glass and silica. Further incorporation of charged stationary phase groups into the organic surface coatings

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resulted in the generation of ion-exchange chromatography supports[2,3]. Studies with 37–74 μm supports showed that proteins could be separated in 20–30 min. It was obvious that microparticulate supports would decrease separation times.

During the studies cited above it was found that clinically significant enzymes could be resolved quickly on ion-exchange columns packed with coated inorganic supports. Since the elution of enzyme from chromatography columns is conventionally monitored by collecting and assaying individual column fractions, the generation of an eluent fraction every 10 sec presented a problem. The need for a continuous monitoring enzyme detector was apparent.

This paper reports studies on the separation of tissue and serum proteins on microparticulate inorganic supports and the development of a fixed time, flow-through enzyme detector that maintains zero-order reaction kinetics with respect to substrate during detection.

EXPERIMENTAL

Apparatus

Liquid chromatography was carried out with a Micromeritics Model 7000 liquid chromatograph with a 254-nm UV detector (Micromeritics, Norcross, Ga., U.S.A.). The Model 705 column packer was obtained from Micromeritics.

Steric exclusion chromatography was on 300 × 4.1 mm I.D. stainless-steel columns. The Partisil PXS 10/25 column was obtained from Whatman (Clifton, N.J., U.S.A.).

The post-column enzyme detector consisted of an Isco Model 384 pumping system (Instrument Specialties, Lincoln, Neb., U.S.A.) with a Perkin-Elmer LC-55 detector (Perkin-Elmer, Norwalk, Conn., U.S.A.). 400 × 4.8 mm I.D. stainless-steel columns were used for the reactor.

Reagents

Glycidoxypropyltrimethoxysilane and triglycidoxyglycerol were obtained from Polyscience (Warrington, Pa., U.S.A.). Controlled porosity glass (CPG) was from Corning (Medfield, Mass., U.S.A.); Lichrospher Si-100 was from EM Labs. (Elmsford, N.Y., U.S.A.); and non-porous sodium silicate was from Whatman. Diethylaminoethanol, magnesium chloride, and nitrophenylphosphate were from Aldrich (Milwaukee, Wisc., U.S.A.). Creatine phosphate, ADP, glucose, NADP, AMP, glutathione, hexokinase, glucose-6-phosphate dehydrogenase, lactate, NAD, bovine serum albumin, calf thymus DNA, bovine spleen deoxyribonuclease II, glycylphenylalanine, cytochrome C, the nucleotides, and alkaline phosphatase were from Sigma (St. Louis, Mo., U.S.A.). The hemoglobin control, HemoControl A_1FSA_2, was purchased from Helena Labs. (Beaumont, Texas, U.S.A.). Rat liver homogenates were provided through the courtesy of Dr. Kim and Dr. Rodwell, Department of Biochemistry, Purdue University.

Procedures

Preparation of columns. Isopropanol was stirred with a magnetic stirring bar in the cylindrical base of the packer. About 2 g of support were added gradually so the stirring bar could be adjusted to keep all the particles in suspension. Then the top,

which had the column attached vertically upwards and an inlet of solvent, was bolted on. Isopropanol was pumped at 2 ml/min until the pressure stabilized. This usually took about 30 min. After packing, a 2-μm column terminator was quickly attached.

Preparation of Glycophase G. Glycophase G was prepared by reacting 10% glycidoxypropyltrimethoxysilane with CPG or silica at 95° for 2 h as described previously[1].

Preparation of DEAE-Glycophase G. DEAE-Glycophase G was prepared by reacting Glycophase G with triglycidylglycerol–diethylaminoethanol–dimethylformamide (2:4:4) as described previously[3]. The resultant support was coated with a 5% solution of triglycidylglycerol and heated at 120° for 1 h. It was then polymerized in a fluidized bed with BF_3 etherate[3] followed by reaction in 1 *N* HCl for 2 h at room temperature. The support was then filtered, washed with water and acetone, and dried.

Preparation of creatine phosphokinase isoenzymes. A mixture containing the three creatine phosphokinase (CPK) isoenzymes was obtained by mixing two partially purified CPK preparations as described previously[3].

Preparation of lactic dehydrogenase isoenzymes. A mixture of lactic dehydrogenase (LDH) isoenzymes was obtained by hybridizing beef heart LDH_1 and rabbit muscle LDH_5 according to the "freeze-thaw" dissociation and recombination method[4]. The resultant solution was dialyzed and concentrated in an Amicon protein concentrator (with PM 30 membrane).

RESULTS

Packing columns

Although both balanced density methods[5–9] and the viscosity method[6] have been employed successfully in packing microparticulate supports, a technique was chosen that did not employ toxic organic solvents or packing pressures three to five times the column operating pressure. Column support material was slurried with isopropanol in a closed stainless-steel cylinder with an unpacked analytical column and solvent inlet line attached. The column was packed by pumping isopropanol through the cylinder and column at 2 cc/min while the support was kept in suspension by magnetic stirring. Packing was usually completed in 30 min with pressures under 3000 p.s.i. Column plate heights (H) of 0.2 mm for glycylphenylalanine in 0.05 *M* KH_2PO_4 buffer, pH 6, were consistently obtained. Unfortunately, plate heights for proteins and other macromolecules are higher than those for small molecules. This is due to the lower diffusion coefficient of proteins, $1–8 \times 10^{-7}$ cm^2/sec, compared with 10^{-5} for an amino acid in water. H is approximately ten times higher for DNA than for glycylphenylalanine.

Steric exclusion chromatography

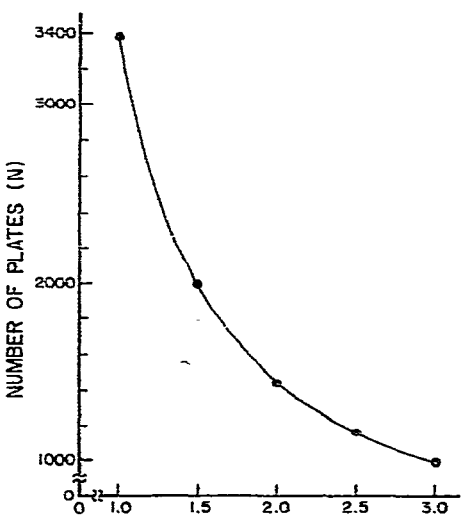
It was noted in the original studies on the separation of proteins with glycerylpropylsilyl-coated controlled porosity glass (Glycophase G/CPG) supports that resolution was inferior to that obtainable on classical carbohydrate gel supports[1]. This difference in relative efficiency influenced the number of theoretical plates required for a separation and thus the column length. In an effort to explain this phenomenon, we will examine the equation that describes resolution.

Previously, we derived a formula which relates resolution, R_s, to the factors important in steric exclusion chromatography[10]

$$R_s = \frac{\sqrt{N}}{2} \frac{pK_D''(\alpha - 1)}{2\alpha + pK_D''(\alpha + 1)}$$

K_D'' is the distribution coefficient of the second solute, N is the number of theoretical plates, α is the separation factor ($\alpha = K_D''/K_D'$), and p is permeability. Permeability equals V_i/V_0, where V_0 is the column void volume and V_i is the volume of solvent contained in the support pores. The permeabilities of controlled porosity inorganic supports range from 0.8–1.2 in contrast to those of carbohydrate supports, which have values of 1–3. Therefore, to achieve a given resolution, a column of inorganic support must possess more plates than a corresponding carbohydrate column of greater permeability. This relationship between permeability and the number of plates is shown in Fig. 1 for a resolution of 1 and a separation factor of 1.25 at a K_D'' of 0.5. A comparison of 550 Å pore diameter Glycophase G/CPG ($p = 1$) and Sephadex G-200 ($p = 2.2$) indicates that 2.6 times as many plates are required for comparable resolution with Glycophase G/CPG.

The utility of microparticulate size discriminating columns was first examined by following the hydrolysis of calf thymus DNA (2,000,000 daltons) with bovine spleen



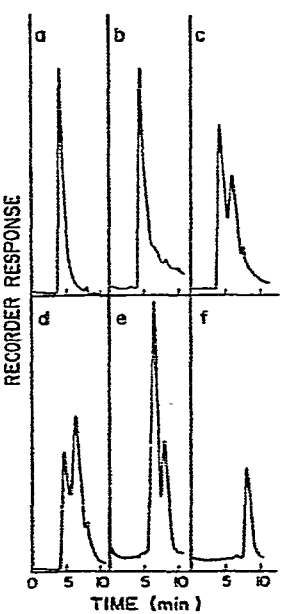


Fig. 1. Effect of column permeability (p) on the number of plates (N) required to give a resolution of 1.0 at an α value of 1.25 at $K_D'' = 0.5$ in steric exclusion chromatography.

Fig. 2. Time study of the digestion of calf thymus DNA by bovine spleen deoxyribonuclease II (DNase). Analysis is on a 300 × 4.1 mm I.D. column packed with Glycophase G/Lichrospher Si-100; (10 μm particle size; 100 Å pore diameter), temperature, 25°. The mobile phase is 0.05 M KH_2PO_4, pH 6; flow-rate, 0.35 ml/min; pressure, 670 p.s.i. DNase was added at room temperature and the solution monitored. (a) Before DNase addition; (b) after 2 min digestion with DNase; (c) after 38 min; (d) after 61 min; (e) after 170 min; (f) after 261 min.

deoxyribonuclease II. This enzyme is an endonuclease that catalyzes the hydrolysis of DNA to oligonucleotides. During this process there are repeated internal cleavages of oligonucleotide fragments with concomitant molecular weight reduction. HPLC makes it possible to assess the molecular weight distribution of products during the course of the enzyme reaction, as shown in Fig. 2.

The utility of microparticulate size separation supports is further demonstrated in the rapid separation of proteins in Fig. 3. Using a column packed with Glycophase G/CPG with 100 Å pore diameter, albumin, cytochrome C, and glycylphenylalanine were separated in less than 90 sec. This could be useful if a crude fractionation of large, intermediate and small molecular weight components is needed.

A comparison of the separation of components of a crude rat liver homogenate on Glycophase G/Lichrospher Si-100 and Glycophase G/Partisil PXS 10/25 is shown in Figs. 4 and 5, respectively. The differences in exclusion limits for these 100 Å and 55–60 Å pore diameter supports are obvious.

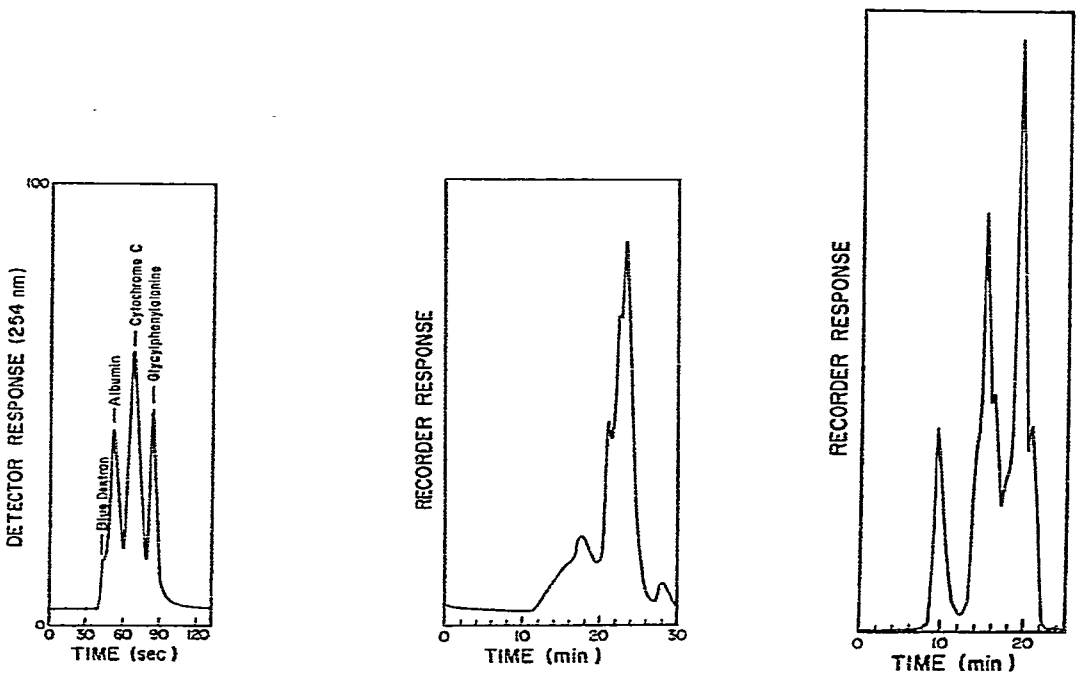


Fig. 3. Steric exclusion chromatography of proteins. Column, 300 × 4.1 mm I.D. stainless steel; packing, Glycophase G/CPG (100 Å pore diameter, 5–10 μm particle size); temperature, 25°. Solvent, 0.1 *M* KH_2PO_4, pH 6; flow-rate, 7 mm/sec; pressure, 2700 p.s.i.

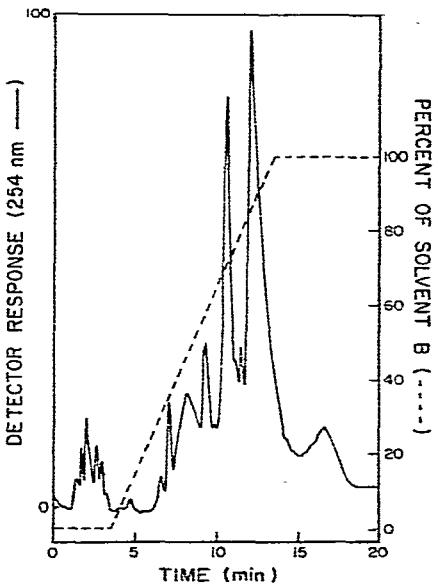
Fig. 4. Size separation of components of a rat liver homogenate. Column, 300 × 4.1 mm I.D. stainless steel; packing, Glycophase G/Lichrospher Si-100 (100 Å pore diameter, 10 μm particle size); temperature, 25°. Solvent, 0.05 *M* KH_2PO_4, pH 6; flow-rate, 0.4 mm/sec (0.18 ml/min); pressure, 250 p.s.i.; detector, 254 nm.

Fig. 5. Size separation of components of a rat liver homogenate. Column, 250 × 4.6 mm I.D. stainless steel; packing, Glycophase G/Partisil PXS 10/25 (55–60 Å pore diameter, 10 μm particle size), temperature, 25°. Solvent, 0.05 *M* KH_2PO_4, pH 6; flow-rate, 0.46 mm/sec (0.19 ml/min); pressure, 480 p.s.i.; detector, 254 nm.

Ion exchange chromatography

The most generally useful ion-exchange stationary phase for the resolution of proteins is a diethylaminoethyl (DEAE) ether derivatized support. For this reason all protein separations on ion exchangers were restricted to DEAE supports in this study.

The elution profiles of a human serum and rat liver homogenate sample on 5–10 μm DEAE-Glycophase/CPG of 250 Å pore diameter is shown in Figs. 6 and 7, respectively. Although no attempt was made to identify specific components in these mixtures, the resolving power of the support is apparent. In the case of serum samples, these profiles could be useful in clinical diagnosis[11].



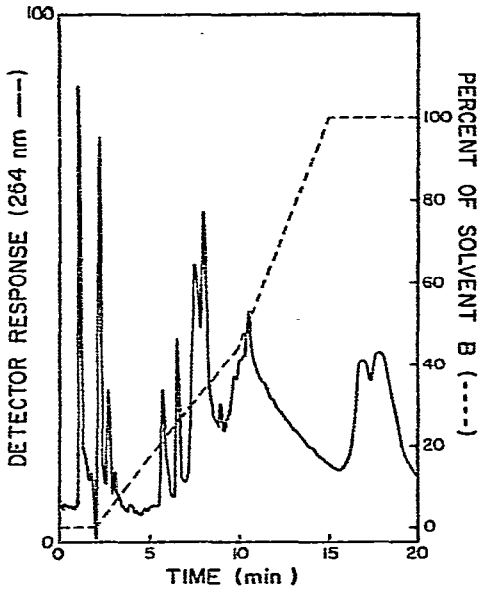
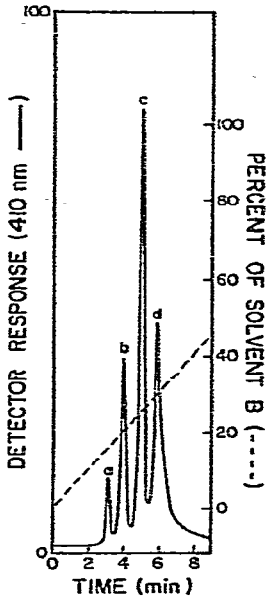


Fig. 6. Separation of human serum proteins on DEAE-Glycophase/CPG. Column, 250 × 4 mm I.D. stainless steel; packing, DEAE-Glycophase/CPG (250 Å pore diameter, 5–10 μm particle size), temperature, 25°. Solvent, (A) 0.02 *M* Tris, pH 8, (B) 0.05 *M* Tris, 0.3 *M* NaCl, pH 7.5; flow-rate, 2 mm/sec (1.5 ml/min); pressure, 1450 p.s.i.; detector, UV, 0.05 a.u.f.s.

Fig. 7. Separation of components of a rat liver homogenate (10,000 *g* supernatant). Column conditions, same as in Fig. 6.

Identification of hemoglobin constituents in blood is useful in diagnosing certain disease states and traits such as sickle cell anemia and thalassemia[12]. Although ion-exchange chromatography gives good qualitative and quantitative separations, its utility is limited due to the slowness of traditional methods[13]. Electrophoresis has become the generally used technique. The resolution of a hemoglobin mixture containing hemoglobins A_1, S, A_2, and F in 6 min is shown in Fig. 8. The identities of the peaks were verified by comparing the retention times with standards. Hemoglobin S exists in the blood of people with the sickle cell trait or disease. Hemoglobin F represents fetal hemoglobin and is found in the blood of newborn



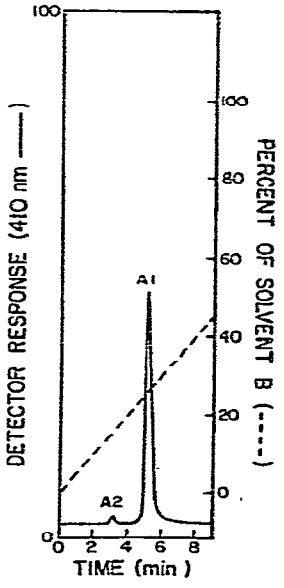


Fig. 8. Separation of a control mixture of hemoglobins (HemoControl A_1FSA_2). Column, 250 × 4 mm I.D. stainless steel; packing, DEAE-Glycophase/CPG (250 Å pore diameter, 5–10 μm particle size), temperature, 25°. Solvents, (A) 0.0125 *M* Tris, pH 8, (B) 0.0125 *M* Tris, 0.15 *M* NaCl, pH 8; flow-rate, 3.33 mm/sec (2.5 ml/min); pressure, 2200 p.s.i. a = Hemoglobin A_2; b = hemoglobin S; c = hemoglobin A_1; d = hemoglobin F.

Fig. 9. Separation of hemoglobins from normal blood cells on DEAE-Glycophase/CPG. Column conditions same as in Fig. 8.

babies. Normal individuals have predominantly hemoglobin A_1 with a small amount of hemoglobin A_2, as shown in Fig. 9.

Separation and detection of CPK and LDH isoenzymes from tissue sources are shown in Figs. 10 and 11, respectively. The detection system used in these studies will be described later in this paper. Isoenzyme separations are of particular clinical significance. In the case of CPK, CPK_2 concentrations in human serum may be used in the diagnosis of myocardial infarction[14,15]. LDH isoenzymes may also be used for diagnosing heart attacks in addition to liver diseases[15].

The DEAE support may also be used to separate small molecules. The resolution of a series of nucleotides on this support is seen in Fig. 12. UDP and ADP co-elute in peak f under the conditions used for this separation. These two nucleotides can be completely separated by eluting isocratically with a buffer system containing 80% of 0.005 *M* KH_2PO_4 buffer (pH 3.35) and 20% of 0.5 *M* KH_2PO_4 buffer (pH 4.1).

The effect of mobile phase velocity on plate height for several DEAE supports of different particle size is shown in Fig. 13. As expected, the microparticulate supports exhibit considerably higher efficiencies at all mobile phase velocities.

Continuous enzyme monitoring

Detection of enzyme activity may be accomplished by incubating an enzyme (E) with an appropriate substrate (S) and monitoring the formation of product (P)

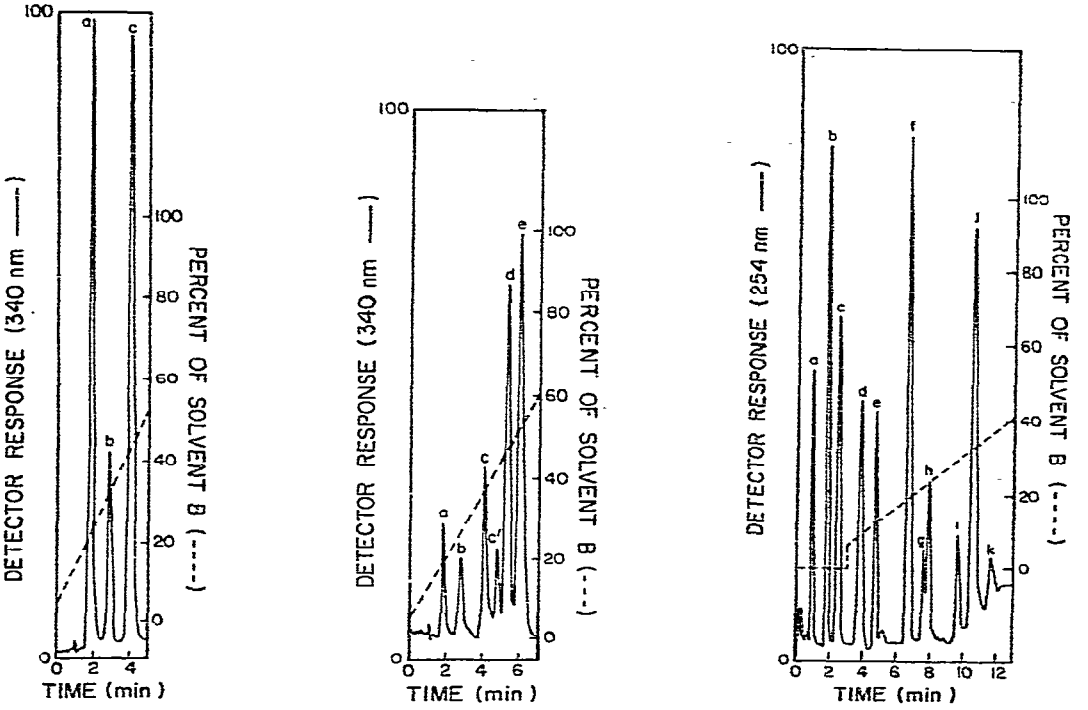


Fig. 10. Separation of CPK isoenzymes using a post-column enzyme detector. Column, 250 × 4 mm I.D. stainless steel; packing, DEAE-Glycophase/CPG (250 Å pore diameter, 5–10 μm particle size), temperature, 25°. Solvents, (A) 0.05 *M* Tris, 0.05 *M* NaCl, 10^{-3} *M* mercaptoethanol, pH 7.5, (B) 0.05 *M* Tris, 0.3 *M* NaCl, 10^{-3} *M* mercaptoethanol, pH 7.5; flow-rate, 4 mm/sec (3 ml/min); pressure, 2500 p.s.i.; a = CPK_3; b = CPK_2; c = CPK_1.

Fig. 11. Separation of LDH isoenzymes using a post-column enzyme detector. Column, 250 × 4 mm I.D. stainless steel; packing, DEAE-Glycophase/CPG (250 Å pore diameter, 5–10 μm particle size), temperature, 25°. Solvents, (A) 0.025 *M* Tris, pH 8.0, (B) 0.025 *M* Tris, 0.2 *M* NaCl, pH 8.0; flow-rate, 4 mm/sec (3 ml/min); pressure, 2500 p.s.i.; a = LDH_5; b = LDH_4; c = LDH_3; d = LDH_2; e = LDH_1.

Fig. 12. Separation of twelve common nucleotides. Column, 250 × 4 mm I.D. stainless steel; packing, DEAE-Glycophase/CPG (250 Å pore diameter, 5–10 μm particle size), temperature, 25°. Solvents, (A) 0.015 *M* KH_2PO_4, pH 3.35, (B) 0.5 *M* KH_2PO_4, pH 4.1; flow-rate, 4 mm/sec (3 ml/min); pressure, 2500 p.s.i.; detector, UV, 0.05 a.u.f.s. a = CMP; b = AMP; c = UMP; d = GMP; e = CDP; f = ADP; g = GDP; h = CTP; i = UTP; j = ATP; k = GTP.

after a fixed time. This obviously could be achieved in a flow-through reactor, as indicated in the analytical system in Fig. 14. The system consists of an analytical column and a packed post-column that is used as an enzyme reaction bed. These two columns are connected by a union tee that also serves as the entrance for substrate (S). After introduction of substrate, the enzyme reaction takes place in the post-column and reaction product(s) are measured spectrophotometrically after elution from the column. On some occasions, one has to employ a series of coupled enzyme reactions to obtain a spectrophotometrically measurable end product. In this case, the solution in the substrate pump has to include all the enzymes and substrates involved in the coupling reactions.

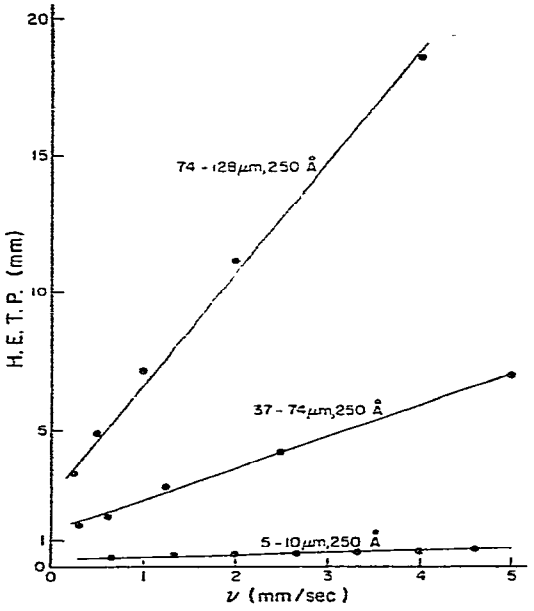


Fig. 13. Effect of mobile phase velocity on efficiency of DEAE-Glycophase/CPG supports.

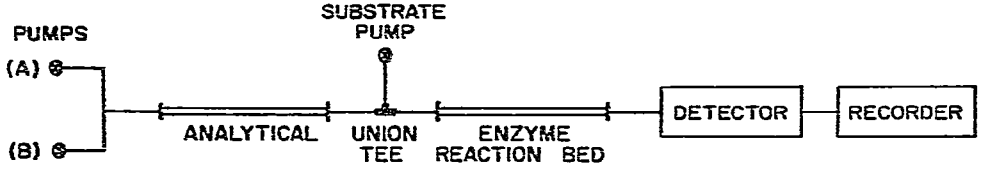


Fig. 14. A diagrammatic representation of the enzyme-monitoring system.

A major problem in the design of a flow-through enzyme detector is the minimization of band spreading in the detector. It may be determined theoretically that non-porous supports will minimize band spreading in a post-column reactor. Additionally it should be noted that any association of enzyme, substrate, or product molecules with the post-column reactor material would result in both demixing of reactants and band spreading.

After the examination of a series of support materials it was determined that non-porous spherical sodium silicate glass beads were the most satisfactory commercially available material for minimizing both band spreading and adsorption. Post-column band spreading was evaluated by comparing chromatograms run with and without the post-column in the system. The comparison of chromatograms of a nucleotide mixture separated by a DEAE-Glycophase/CPG column of 5–10 μm particle size in the presence and absence of a post-column is seen in Fig. 15. It was concluded that this amount of band spreading was acceptable for most applications.

It is well known that product formation in an enzyme reaction is dependent on temperature, pH, substrate concentration, and time. When all of these variables are fixed in the post-column reactor, the detector is linear, as indicated in Fig. 16.

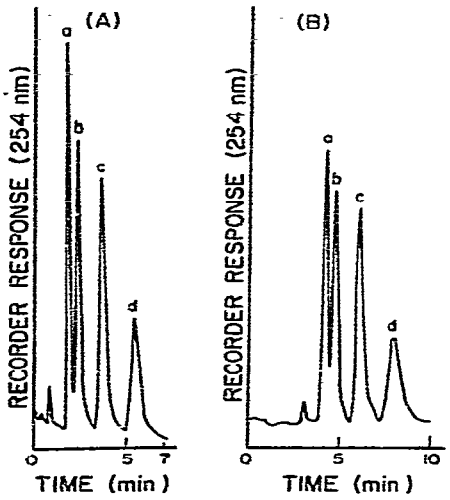


Fig. 15. Band spreading of a nucleotide mixture in a post-column packed with non-porous support. (A) An analytical column 250 × 4 mm I.D.; packed with DEAE-Glycophase/CPG (5–10 μm particle size, 250 Å pore diameter), temperature 25°, was connected directly to a UV_{254} detector. (B) A post-column 600 × 4.8 mm I.D. packed with 40-μm non-porous sodium silicate glass, temperature 25°, was inserted between the analytical column and the detector. The sample was eluted isocratically with 0.015 *M* KH_2PO_4 buffer (pH 3.35) at a mobile phase velocity of 3.33 mm/sec. a = CMP, b = AMP, c = UMP, d = GMP.

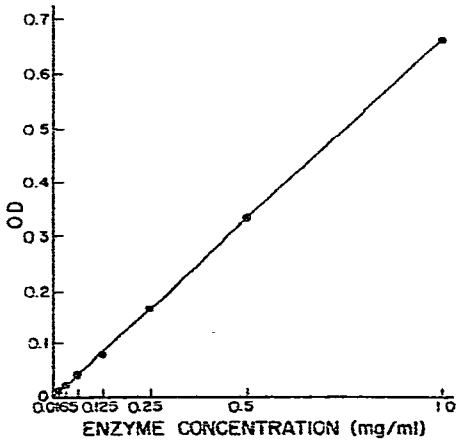


Fig. 16. Linearity of flow-through enzyme detector. Operational parameters are as follows: sample, hog intestinal alkaline phosphatase; buffer, 0.005 *M* borate buffer (pH 8) with 2 m*M* $MgCl_2$ and 0.3 *M* NaCl; analytical column, 600 × 4.8 mm I.D., packed with DEAE-Glycophase/CPG (37–74 μm particle size, 250 Å pore diameter); post-column, 400 × 4.8 mm, packed with Glycophase G/non-porous sodium silicate (40 μm particle size). Substrate, 4 m*M* *p*-nitrophenyl phosphate in the above buffer; flow-rate, (a) 1.37 ml/min (analytical pump), (b) 0.70 ml/min (substrate pump); pressure 300 p.s.i.; detector, 410 nm; at room temperature.

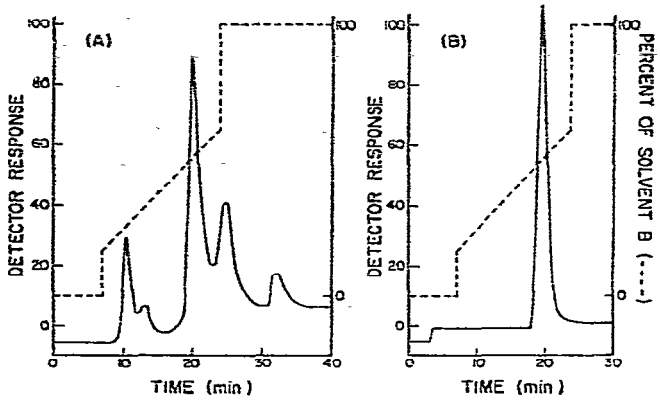


Fig. 17. Fractionation of a commercial calf intestinal alkaline phosphatase sample with and without the enzyme detector. Operational parameters: sample, (A) 8 mg/ml calf intestinal alkaline phosphatase, (B) 0.4 mg/ml calf intestinal alkaline phosphatase; buffer, initial buffer 0.05 *M* Tris (pH 8), final buffer 0.05 *M* Tris (pH 8), 0.3 *M* NaCl. Analytical column, 500 × 5 mm I.D., packed with DEAE-Glycophase/CPG (37–74 μm particle size, 250 Å pore diameter), temperature, 25°; post-column, 600 × 5 mm I.D., packed with Glycophase G/non-porous sodium silicate (40 μm particle size), temperature, 25°. Substrate, 8 m*M* *p*-nitrophenyl phosphate in initial buffer; flow-rate, (a) 1.37 ml/min (analytical pump), (b) 0.35 ml/min (substrate pump); pressure 300 p.s.i.; detector, (A) 280 nm, (B) 410 nm; temperature, room temperature.

When operating in the enzyme detection mode the instrument is absolutely specific for a single enzyme or group of isoenzymes. Fig. 17A is a UV tracing of commercial calf intestinal alkaline phosphatase. Fig. 17B is the enzyme activity tracing indicating that only a single component in the mixture has alkaline phosphatase activity. It should also be noted that the enzyme detector is approximately 200 times more sensitive in the case of alkaline phosphatase.

The use of this enzyme detector in the determination of CPK and LDH isoenzyme profiles has already been shown in Figs. 10 and 11.

CONCLUSIONS

It may be concluded that hydrophilically coated microparticulate inorganic supports may be used in the high-performance separation of proteins. The separation of most protein mixtures could be achieved in 10 min or less. Separation times are generally 10–100 times shorter than those of classical carbohydrate gel supports.

The continuous monitoring enzyme detector provides a simple, high-sensitivity, linear detection system for enzymes with little band spreading. It is probable that a combination of microparticulate analytical columns and enzyme-specific detectors will be of considerable utility in the qualitative and quantitative analyses of proteins in clinical and research laboratories.

ACKNOWLEDGEMENT

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