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MICROPREPARATIVE PROTEIN PURIFICATION BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Micropreparative purification of the four subunits of phosphorylase kinase (molecular weights 16,680, 43,000, 113,000 and 132,000) by reversed-phase high-performance liquid chromatography has provided quantities sufficient for some of the first structural studies of these proteins. The best yield from a 25 × 0.46 cm I.D. column was obtained on a packing material (5-μm C_{18}) bonded under conditions resulting in a relatively low ligand density; of a total 250 μg of protein applied, 76% was recovered. Low recoveries from 5–30-μg sample loads suggest partial irreversible adsorption. Retention of protein by the column after an initial micropreparative separation adversely affects resolution and recovery on subsequent separations. Incorporation of gradient wash steps between sample injections was necessary to maintain column performance and to prolong usable column lifetime. The use of a short column with a large diameter (6 × 1 cm) gave increased loading capacity above 400 μg protein and enhanced recovery while maintaining good resolution. A less expensive reversed-phase support (17-μm C_{18}) provided adequate resolution for this separation.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) has become one of the most versatile fractionation methods available to the protein chemist. The use of RP-HPLC for the purification of small[1] as well as large peptides[2] and hydrophobic, membrane-associated polypeptides[3] is now well documented[4]. However, HPLC micropreparative purification of proteins, particularly large proteins, in quantities sufficient for further studies remains a challenge. For the optimum RP-HPLC separation of large peptides and proteins it is clear that large-pore diameter

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supports are superior[5–8]. Recently it has been reported that the silica type used for the reversed-phase support is also of paramount importance to protein resolution[9]. Using large-pore diameter superior-grade reversed-phase silica we have developed an HPLC micropreparative purification of the subunits of a large oligomeric protein and carried out structural studies of the subunits. This report examines the resolution, recovery and loading capacity of rabbit muscle phosphorylase kinase on various reversed-phase supports in columns of different length and diameter.

Phosphorylase kinase is a key multiple-activity regulatory enzyme involved in glycogen metabolism (for reviews see refs. 10 and 11). The rabbit muscle holoenzyme is a large, highly aggregated molecule estimated[12,13] to have a molecular weight of $1.2 \cdot 10^6$–$1.3 \cdot 10^6$ and thought to be composed of four monomers of about 300,000 molecular weight[13]. Each monomer contains in an apparent equimolar ratio four non-identical, tightly associated subunits[13] designated as alpha (molecular weight, M_r = 132,000), beta (M_r = 113,000), gamma (M_r = 43,000) and delta (M_r = 16,680). The delta subunit has been purified by heat precipitation of the other subunits and identified by sequence analyses as the heat-stable Ca^{2+}-dependent modulator protein calmodulin[14]. The larger subunits have proven to be more difficult to separate preparatively. Previously, the only methods that have been successfully used to purify all four subunits were either selective precipitation of beta with lithium bromide, followed by gel filtration in the presence of sodium dodecyl sulfate (SDS)[15], or a combination of gel filtration, hydroxylapatite chromatography and preparative polyacrylamide gel electrophoresis, all in the presence of SDS[16]. Overall, these earlier purification methods are slow, require the presence of a detergent and give low yields.

MATERIALS AND METHODS

Materials

Sequenal-grade trifluoroacetic acid (TFA), triethylamine, pyridine, fluoraldehyde, amino acid and phenylthiohydantoin (PTH) amino acid standards were obtained from Pierce. Phenylisothiocyanate was purchased from Beckman Instruments. Acetonitrile, benzene, ethyl acetate, heptane, methanol and tetrahydrofuran were obtained from Burdick & Jackson Labs. All water for sequencing or HPLC purposes was glass-distilled.

HPLC columns

All protein separations were done on large-pore diameter (300–330 Å) reversed-phase silica. The 5-, 10-, and 17-μm Vydac C_4, C_8, C_{18} and phenyl columns (5 × 0.46 cm I.D., 25 × 0.46 cm I.D. and 6 × 1 cm I.D.) were supplied by the Separations Group (Hesperia, CA, U.S.A.) and the 10-μm Brownlee MPLC Aquapore RP300 columns (25 × 0.46 cm I.D. and 3 × 0.46 cm I.D.) were obtained from Kontron (München, F.R.G.). Both OPA-derivatized amino acids and PTH-amino acids were separated on Beckman Ultrasphere ODS columns (25 × 0.46 cm I.D.).

Preparation of phosphorylase kinase

The enzyme was purified from rabbit muscle according to Cohen[13] with the modification of Jennissen and Heilmeyer[17]. Protein concentrations were determined by either an autoanalyzer, according to Lowry *et al.*[18] or by amino acid analysis.

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis in the presence of SDS was performed according to Fairbanks *et al.*[19] on polyacrylamide slab gels.

Amino acid analysis

Two methods of amino acid analysis were utilized, ion-exchange chromatography with post column ninhydrin derivatization according to Moore and Stein[20] and reversed-phase HPLC with precolumn *o*-phthaldialdehyde (OPA) derivatization essentially as described by Jones *et al.*[21]. Ion-exchange analyses were performed on a Dionex amino acid analyzer Model D-500. Cysteine was determined as cysteic acid[22] and tryptophan from methanesulfonic acid hydrolysates[23]; both were quantified by RP-HPLC with precolumn OPA derivatization.

Quantification of recovery

All protein recoveries were quantified by amino acid analysis (primarily by RP-HPLC with precolumn OPA derivatization) and are based on a molecular weight of $1.22 \cdot 10^6$ for holophosphorylase kinase and a subunit stoichiometry of $(\alpha\beta\gamma\delta)_4$.

Measurement of protein-bound phosphate

Protein-bound phosphate was measured essentially by the method of Stull and Buss[24] with the modifications previously described[25]. Samples containing 0.1–4.5 nmol phosphate and 5–437 μg protein were dried directly from the HPLC column eluate for protein and phosphate determinations. Protein concentrations were determined by amino acid analyses using RP-HPLC and precolumn OPA derivatization.

Microsequence analysis

Tarr manual Edman degradations of the alpha, beta and gamma subunits from rabbit muscle phosphorylase kinase were carried out on 750–2000 pmol of the intact unmodified proteins. Sequencing methodology was as previously described by Crabb and Saari[26] except for the modifications recently described[25]. PTH-amino acids were identified and quantified at 269 nm by both gradient[27] and isocratic[28] HPLC methods.

Chromatography

Proteins as well as PTH-amino acids were chromatographed on a Kontron Model 427 HPLC system equipped with a Uvikon 722 spectrophotometer and a 2-pen Kipp & Zonen chart recorder. Tryptophan fluorescence was monitored at 285 nm excitation and 340 nm emission with a Kontron Model SFM 23LC spectrofluorometer.

Amino acid analysis by HPLC was performed with a Laboratory Data Control (LDC) gradient HPLC system equipped with a LDC Model 301 integrator, a Kontron Model 200 programmer and the Kontron Model SFM 23LC spectrofluorometer (340 nm excitation, and 455 nm emission).

RESULTS AND DISCUSSION

Phosphorylase kinase subunit separation and structural characterization

RP-HPLC of holophosphorylase kinase rapidly separates all four subunits (Fig. 1). Either gradient program described in the legend to Fig. 1 works well. However, the step gradient incorporated in Fig. 1A provides a greater separation of alpha and beta. Electrophoresis in the presence of SDS (Fig. 2) reveals a single band for all the HPLC purified subunits except alpha which migrated with the alpha isozyme, alpha prime. The separation provides a complementary analytical method to SDS–PAGE, the sensitivity of which can be enhanced by fluorescence detection of tryptophan residues.

Micropreparative purification of the subunits by RP-HPLC has provided quantities sufficient for some of the first structural characterizations of these proteins. The first amino-terminal sequence analyses of the alpha, beta and gamma subunits were carried out by Tarr manual Edman degradation[25]. A comparison of the amino-terminal sequences of the gamma and alpha subunits with two other protein kinases[29,31] is shown in Table I. The homology between gamma and the catalytic subunit of cAMP-dependent protein kinase is now apparent from the complete amino acid sequence of gamma[30]. For alpha, additional structural information is required to evaluate whether a homologous relationship exists with the transforming protein from the Rous sarcoma virus. The beta subunit has a blocked amino-terminus.

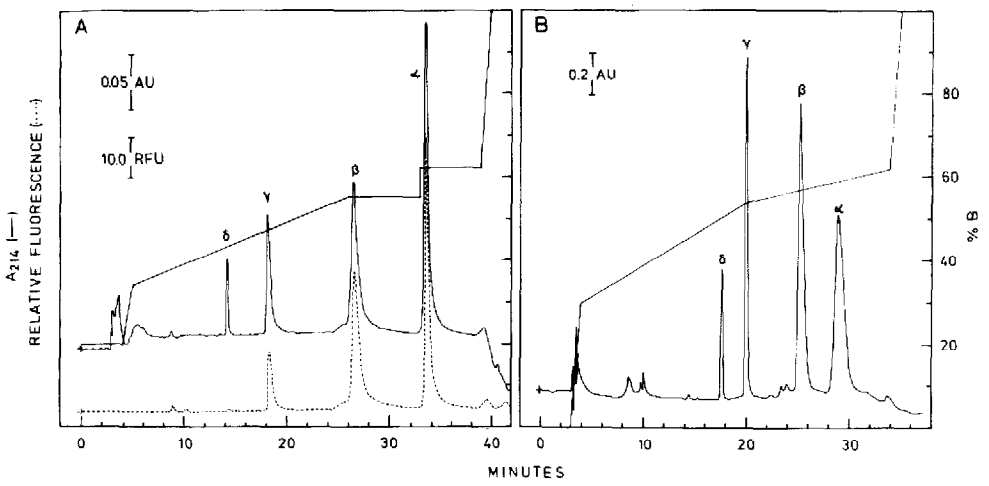


Fig. 1. RP-HPLC of rabbit muscle phosphorylase kinase. Enzyme was injected in 50 m*M* sodium glycerol-2-phosphate pH 7, containing 2 m*M* EDTA, 1 m*M* dithioerythrotol and 50% glycerol, and the gradients were developed at a flow-rate of 1 ml/min with 0.1% TFA in water as solvent A and 100% acetonitrile containing 0.08% TFA as solvent B. Absorbance was monitored at 214 nm (2.0 AUFS) and tryptophan fluorescence at 285 nm excitation, 340 nm emission (100% low sensitivity). Profile A: 30 μg of enzyme was applied to a 5-μm Vydac C_{18} column (25 × 0.46 cm I.D.) at 20% solvent B and the following gradient was developed: 20–34% B in 1 min, starting at time 1 min, then 34–55% B in 21 min, isocratic 55% B for 7 min, then step to 62% B at time 30 min, and 62–100% B in 1 min at time 36 min. Profile B: 300 μg of enzyme was applied to a 5-μm Vydac C_4 column (25 × 0.46 cm I.D.) at 0% solvent B and the following gradient was developed: 0–30% B in 1 min at time 0, then 30–54% B in 16 min, 54–62% B in 14 min, and 62–100% B in 1 min at time 31 min.

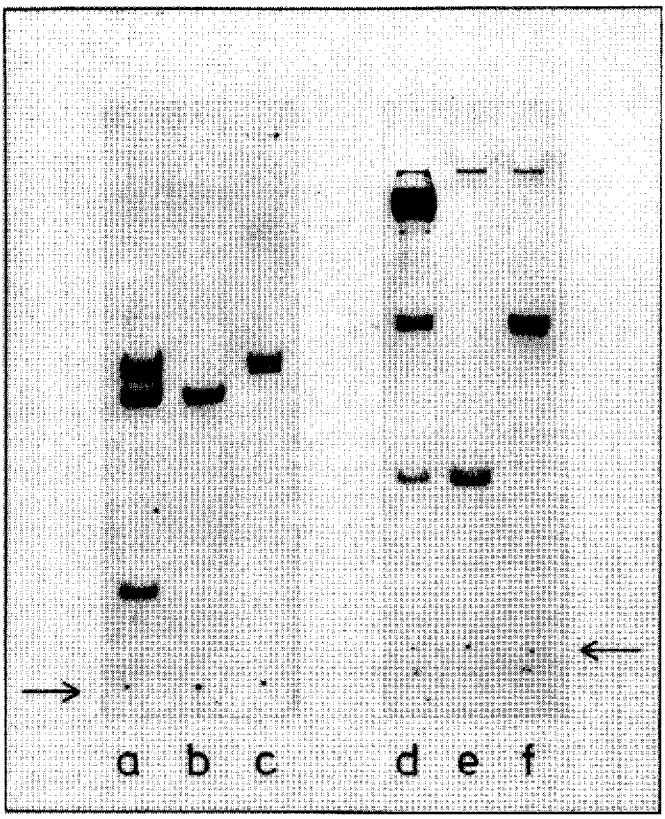


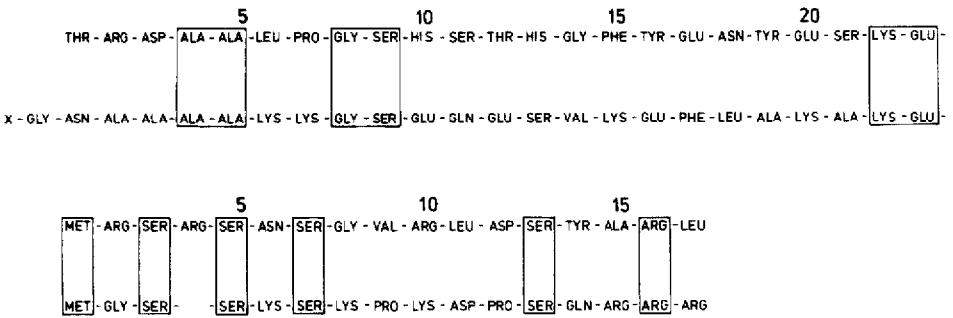
Fig. 2. SDS–PAGE (17) on 4% acrylamide (a–c) and 10% acrylamide (d–f) slab gels. The samples were analyzed at the protein levels indicated: (a) and (d), holophosphorylase kinase, 10 μg and 20 μg, respectively; (b) the HPLC-purified beta subunit, 5 μg; (c) the HPLC-purified alpha subunit, 4 μg; (e) the HPLC-purified delta subunit, 4 μg; (f) the HPLC-purified gamma subunit, 5 μg.

The first protein-bound phosphate analyses of the subunits were possible as a result of micropreparative purification by HPLC[25]. RP-HPLC of proteins in a volatile, phosphate-free solvent system provides a relatively gentle method of removing unbound phosphate; the samples may be recovered directly from the column eluate for both protein and phosphate determinations without the previously recommended hot (90°C) trichloroacetic acid (TCA) treatment[24]. The hot TCA treatment may hydrolyze, in addition to acid-labile phosphohistidine and phospholysine, other phosphorylated residues exhibiting unusual lability owing to their particular environment in the protein.

The amino acid compositions of the HPLC-purified subunits were determined and represent the first complete amino acid analyses of the alpha and beta subunits[25]. The sum of these individual subunit compositions are in excellent agreement with analyses of the holoenzyme previously reported[12,13]. Relative subunit molecular weights, as determined by SDS–PAGE in 4, 5, 7 and 10% acrylamide, were: alpha 132,000, alpha prime 127,000, beta 113,000 and gamma 43,000[25].

TABLE I

COMPARISON OF AMINO-TERMINAL SEQUENCES



* Ref. 29.
** Ref. 30.

Resolution and recovery

Micropreparative purification of the subunits was carried out by repetitive RP-HPLC. To columns of 4.6 mm diameter, sample applications greater than about 300 μg of holoenzyme resulted in partial contamination of beta with alpha and lower yields of all the subunits. The best yields from a 25 × 0.46 cm I.D. column without contamination of beta with alpha were obtained on 5-μm Vydac C_{18} (lot No. 1631). Total protein recovery from 250 μg of holophosphorylase kinase was about 76% with individual subunit recoveries of delta 70%, gamma 79%, beta 88% and alpha 66%. A comparison of recoveries from 250-μg, 500-μg and 1.0-mg sample loads is shown in Fig. 3. Although the subunits are well resolved on a variety of Vydac reversed-phase supports (including 5- and 10-μm C_4, C_8, C_{18} and phenyl), lower recoveries have been obtained from 25 × 0.46 cm columns filled with reversed-phase material other than lot No. 1631 (Table II). The apparent superiority of lot No. 1631 with regard to recovery may be related to the bonding conditions and ligand density. The manufacturer indicates that lot No. 1631 was bonded under different conditions than those used for the other supports in this work, resulting in essentially a monomeric coating of C_{18} and a relatively lower ligand density[32].

The recovery from analytical sample loads was also examined. Fig. 4 shows the absorbance profiles obtained from RP-HPLC of 5–30-μg loads of phosphorylase kinase as well as the absorbance and tryptophan fluorescence profiles from one blank gradient elution immediately following the initial separations. Although the tryptophan fluorescence profiles demonstrate that desorption of the sample was not complete on any of the gradient elutions, absorbance suggest that very little protein remains on the column. The total recovery from the 65 μg of protein chromatographed in Fig. 4 ranged from 58% for the 5-μg load to 55% for the 30-μg load and overall averaged 55% (Table II). It should be noted in Table II that an average protein recovery of 50% was obtained from about 1.5 mg of phosphorylase kinase

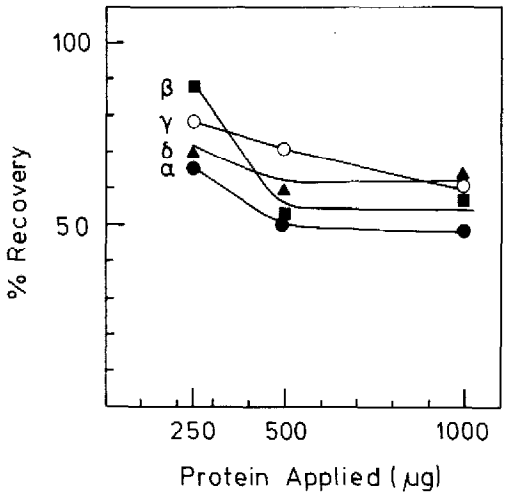


Fig. 3. Recovery of the alpha (●), beta (■), gamma (○) and delta (▲) subunits of phosphorylase kinase from RP-HPLC on a 5-μm Vydac C_{18} column (25 × 0.46 cm I.D.), lot 1631. Conditions are as in Fig. 5.

TABLE II

PROTEIN RECOVERY

Reversed-phase support	*Column dimensions and particle size*	*Total protein applied (μg)*	*Number of separations*	*Average load (μg)*	*Recovery (%)**				
					Total protein	*Delta 16,700***	*Gamma 43,000***	*Beta 113,000***	*Alpha 132,000***
C_4 Vydac TP214 lot No. 1830	25 × 0.46 cm, 5 μm	1558	8	195	50	54	55	45	45
C_4 Vydac TP214 lot No. 150424	25 × 0.46 cm, 5 μm	65	4	16	55	71	67	53	31
C_4 Vydac TP214 lot No. 150424	6 × 1 cm, 5 μm	3800	9	422	76	89	79	71	64
Aquapore RP300 MPLC guard column	3 × 0.46 cm, 10 μm	276	3	92	43	49	51	33	38

* Based on amino acid analysis.
** Subunit molecular weights.

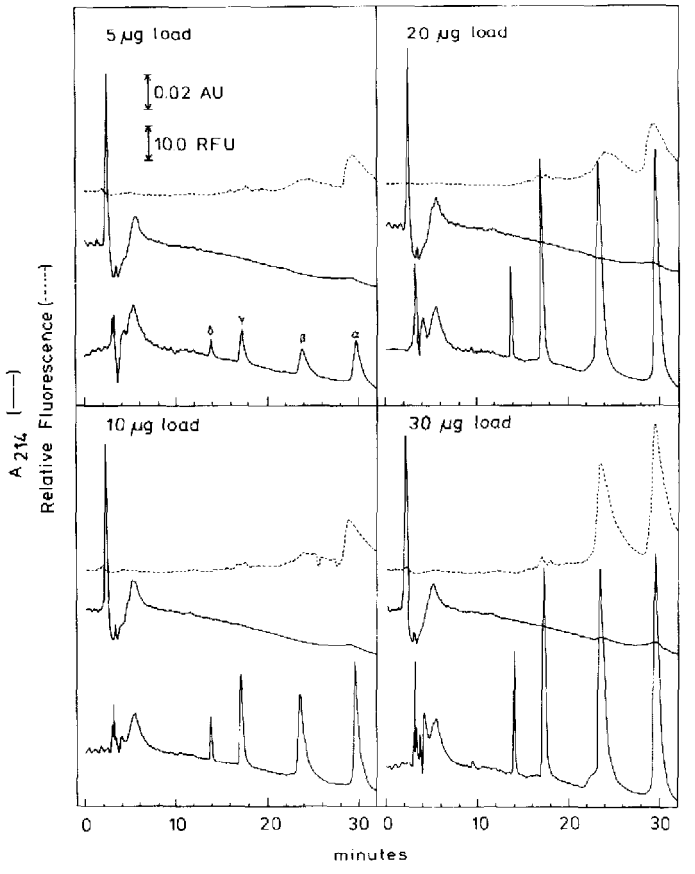


Fig. 4. HPLC of 5–30 μg of phosphorylase kinase on a 5-μm Vydac C_4 column (25 × 0.46 cm I.D.), lot 150424. The absorbance and tryptophan fluorescence profiles from the first blank gradient elution immediately following the initial separations are shown above each initial separation. All profiles are recorded on the same scale for absorbance (0.2 AUFS) and fluorescence (50% high sensitivity). Conditions are essentially as in Fig. 1A. Based on a stoichiometry of $(\alpha\beta\gamma\delta)_4$, each sample load contains about 5.5% delta, 14% gamma, 37% beta and 43% alpha. Delta does not contain tryptophan [14].

on another Vydac C_4 support (lot No. 1830) during eight micropreparative separations. These recovery values are similar to those reported by Pearson *et al.*[9], namely that from a Vydac C_8 column (25 × 0.41 cm I.D.) saturated with 21 mg of ovalbumin, 60% was recovered on the first gradient cycle, while the remaining 40% was recovered with the subsequent eleven gradients. Whether a large number of gradients could elute 100% of the analytical sample loads applied in Fig. 4 or whether the protein is in part irreversibly adsorbed by the column is not known. However, partial irreversible adsorption seems likely.

Column loading, length, diameter and particle size

A major factor influencing column loading during micropreparative protein purification is the amount of protein retained by the column after the initial separation. Fig. 5 shows the desorption of the subunits during five blank gradient elutions

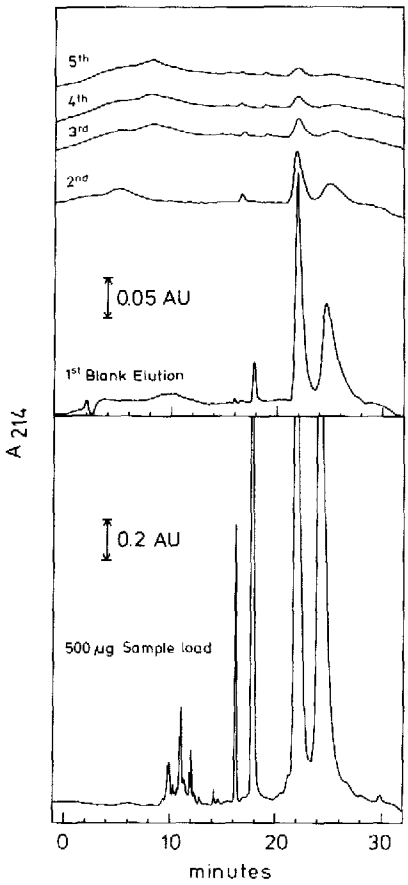


Fig. 5. HPLC of 500 µg of phosphorylase kinase on a 5-µm Vydac C_{18} column (25 × 0.46 cm I.D.), lot 1631, and consecutively, the five blank gradient elutions immediately following the initial separation. Conditions are as in Fig. 1B. The gradient program was started 8 min after injection and was 0–46% in 1 min at time 0 then 46–68% B in 15 min, 68–75% in 9 min and to 100% B at time 25 min.

after the initial chromatography of 500 µg of phosphorylase kinase. As expected, the larger subunits are the slowest to be desorbed. Without precautionary measures, this well-recognized memory effect[9,33] can severely reduce resolution and recovery on subsequent separations and greatly limit the usable lifetime of the column. To maintain resolution, recovery and column life during purification of the phosphorylase kinase subunits we (a) limit sample loads to 300 µg for 4.6-mm diameter columns and 500 µg for 1-cm diameter columns, (b) routinely utilize a short (7–8 min) gradient from 35% to 65% B as a wash step between each preparative separation and (c) carry out a series of gradient washes after every three to four sample applications. Excessive application of proteins of low solubility in the eluent should be avoided. Sample loads of phosphorylase kinase greater than about 1 mg can precipitate essentially as a protein plug on a 4.6-mm diameter column and, if not physically removed, result in damage to the column from extremely high back pressure.

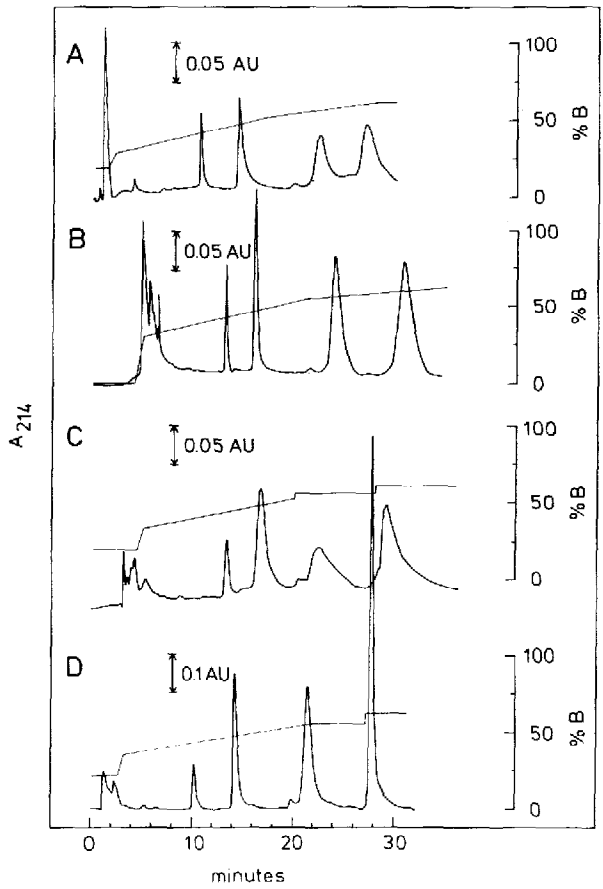


Fig. 6. HPLC of phosphorylase kinase on several reversed-phase supports. (A) 45 μg of enzyme on a 10-μm Aquapore RP 300 MPLC guard column (3 × 0.46 cm I.D.); (B) 60 μg of enzyme on a 10-μm Aquapore RP300 MPLC column (25 × 0.46 cm I.D.) coupled with an Aquapore RP300 MPLC guard column (3 × 0.46 cm I.D.); (C) 110 μg of enzyme on a 17-μm Vydac C_{18} column (25 × 0.46 cm I.D.); and (D) 500 μg of enzyme on a 5-μm Vydac C_4 column (6 × 1 cm I.D.). Enzyme was injected in buffer and the indicated gradients were developed with the solvents described in the legend to Fig. 1. The flow-rate was 1 ml/min for all separations except D where it was 3 ml/min.

Column length has been shown to be relatively unimportant for protein separations[7,9]. We have found this to be true for the separation of the phosphorylase kinase subunits; columns 3–6 cm in length resolve the subunits essentially as well as the more expensive 25-cm columns. A comparison of the separation obtained on a 3-cm guard column (MPLC Aquapore RP300) with that obtained on a 25-cm column plus the guard column is shown in Fig. 6 (A and B). Recovery from this guard column was relatively low; of 192 μg protein applied in three injections, 43% was recovered (Table II).

With the hope of increasing loading capacity while maintaining good resolution and recoveries we examined the performance of a short column with a large diameter. RP-HPLC of 500 μg of phosphorylase kinase on a 6 × 1 cm column is shown in Fig. 6D. The higher flow-rate (3 ml/min) required to maintain peak shape

and short separation times did not increase back pressure. Total recovery (Table II) from nine injections of phosphorylase kinase applied in an average load of 422 μg was 76%; subunit recovery ranged from 64% to 89%. Compared with the protein recovery from the same or similar reversed-phase material in 25 × 0.46 cm columns (Table II), the larger-diameter column provided an overall increase in yield of 20–25%. Although larger sample loads can be applied to the 6 × 1 cm column, applications of phosphorylase kinase above 500 μg result in partial contamination of beta with alpha.

In the light of the excellent resolution of the subunits on reversed-phase material of 5- and 10-μm particle size, we explored the potential use of a less expensive, larger particle size reversed-phase support. The separation of the subunits on a 17-μm Vydac C_{18} is shown in Fig. 6C. As expected, the peaks are broader. However, adequate resolution of the subunits is obtained, indicating that for certain micropreparative purposes the most expensive supports are not required.

CONCLUSIONS

Micropreparative purification of large proteins by RP-HPLC can result in quantities sufficient for further studies provided precautionary measures are taken to preserve resolution, recovery and column life. For the HPLC purification of the phosphorylase kinase subunits precautionary measures include the avoidance of excessive sample loads and the routine incorporation of gradient wash steps between sample injections. In the present work, a short column with a large diameter (6 × 1 cm) has increased both protein loading and recovery compared with the performance of the same type of support in a longer but narrower column (25 × 0.46 cm). Larger particle size reversed-phase supports appear to provide a less costly yet adequate means for certain HPLC protein separations.

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