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Alternative mobile phases for the reversed-phase high-performance liquid chromatography of peptides and proteins

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ABSTRACT

The use of a high content of acetic acid as mobile phase additive for the reversed-phase high-performance liquid chromatography (RP-HPLC) of several proteins and extracts of biological tissues was evaluated for a divinylbenzene (DVB)-based stationary phase, and the separations obtained with acetic acid gradients in acetonitrile, isopropanol or water were compared with classical polypeptide RP-HPLC on silica C_4 with trifluoroacetic acid (TFA)–acetonitrile. The separation patterns for recombinant derived interleukin-1β (IL-1β) on the C_4 column eluted with TFA–acetonitrile and the DVB column eluted with acetic acid–acetonitrile were similar, but only the polymeric column was able to separate the components present in an iodinated IL-1β preparation. Neither eluent had any harmful effect on the biological activity of IL-1β isolated after RP-HPLC.

Several standard proteins could be separated when the polymeric column was eluted with acetic acid gradients in acetonitrile, isopropanol or water and, although the separation efficiency with acetic acid in water was lower than that in combination with classical organic modifiers, insulin, glucagon and human growth hormone (hGH) were eluted as sharp, symmetrical peaks. The recoveries of insulin and hGH were comparable for all three mobile phases (80–90%).

The separation patterns obtained from a crude acetic acid extract of a normal and a diabetic, human pancreas analysed using acetic acid gradients with or without organic modifiers were found to be similar and comparable to those obtained on a silica C_4 column eluted with an acetonitrile gradient in TFA. The principal differences resulted from the use of different UV wavelengths (215 nm for TFA–acetonitrile, 280 nm for acetic acid).

Acetic acid extracts of recombinant derived hGH-producing *Escherichia coli* were separated on the DVB column eluted with an acetic acid gradient in water. Although the starting material was a highly complex mixture, the hGH isolated after this single-step purification was surprisingly pure (as judged by sodium dodecyl sulphate–polyacrylamide gel electrophoresis).

Consequently several (pure) polypeptides and complex biological samples were separated on a polymeric stationary phase eluted with acetic acid gradients in water without the use of organic modifiers.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is based on modulation of the hydrophobic binding forces between a stationary phase and

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hydrophobic domains in the sample molecules. The sample molecules are bound to alkyl or phenyl groups anchored to a solid matrix, and the chromatographic process is performed by adding substances with greater affinity for these ligands to the mobile phase. Organic modifiers such as acetonitrile, methanol, ethanol or propanol have been the most popular mobile phase additives, but the use of butanol, tetrahydrofuran and acetone has also been reported.

In RP-HPLC peptide and protein analyses, the binding between the ligand and the hydrophobic domains can often be eliminated with a narrow shift in the concentration of organic modifiers and at a relatively low level of these modifiers (*e.g.*, less than 50% acetonitrile) and if no secondary binding phenomena are involved, the chromatography may result in elution of the polypeptide molecules in narrow peaks with good recoveries.

With increasing hydrophobicity of protein molecules, the binding forces gradually increase to the point where addition of up to 90% of organic modifier is insufficient for elution from the stationary phase. Such insufficient mobile phase competition normally leads to chromatograms that show broad peaks with non-ideal shapes, in combination with severely reduced recoveries, a situation often seen after RP-HPLC of membrane proteins.

We have recently published an RP-HPLC analysis of sodium dodecyl sulphate (SDS)-solubilized membrane proteins from human erythrocyte ghosts using a polymeric stationary phase with phenyl ligands, eluted with acetonitrile–acetic acid [1]. Several membrane proteins with molecular weight (MW) $>100\,000$ dalton were eluted with very good recoveries, and this observation lead us to the hypothesis that less hydrophobic sample molecules, *e.g.*, small proteins, could perhaps be eluted at low modifier concentrations from selected reversed-phase stationary phases after addition of acetic acid to the mobile phase, perhaps even without the use of organic modifiers.

In this work, we characterized a polymeric phenyl-based stationary phase using gradients of acetic acid in water, isopropanol or acetonitrile. A number of polypeptides, and also highly heterogeneous extracts of biological tissues, were eluted without the use of classical organic modifiers and the recoveries and biological activities of selected polypeptides were measured. The separation pattern without organic modifier was compared with that obtained after eluting similar samples from a classical silica-based stationary phase (Nucleosil C_4) with a widely used mobile phase [trifluoroacetic acid (TFA)–acetonitrile].

EXPERIMENTAL

HPLC

Commercially available HPLC equipment and columns were used throughout.

Pumps. M6000A, M510, M45, M590 (Waters Assoc.), Spectra-Physics SP 8700 and Gynkotek 300C pumps were used.

Sample injectors. U6K, WISP 710A and 712B (Waters Assoc.), and Model 7125 (Rheodyne) types were employed.

UV-detectors. M440 Lambda Max (Waters Assoc.), Hitachi L4200, Linear UVIS 200 and Pye Unicam UV detectors were used.

Integrators. These were M730, M840 (Waters Assoc.), Hitachi L2500 and Hewlett-Packard 3390A.

Gradient Controllers. M660, M720 and M840 (Waters Assoc.), Gynkotek 250B and Spectra-Physics SP8700 were used.

Chemicals. Acetonitrile, methanol and isopropanol were obtained from Rathburn (HPLC grade S), acetic acid (analytical-reagent grade) from Merck. All other chemicals were of analytical-reagent, sequential or similar purity. Water was obtained from a Millipore Milli-Q apparatus. All mobile phases were filtered through a 0.45-μm Millipore filter and degassed before use. During chromatography, the mobile phases were degassed continously with helium sparkling or by passage through an ERMA ERC 3310 degasser.

Columns. A TSK Phenyl 5PW RP+ column (75 × 4.0 mm I.D.) was obtained from Tosoh, a Nucleosil 300 Å C_4 (5 μm) column (250 × 4.0 mm I.D.) from Macherey-Nagel and a Pentax PEC 102 column (100 × 7.5 mm I.D.) from Pentax. Dynosphere PD-102-RE was used in prepacked columns (250 × 4.0 mm I.D.) or obtained as a packing material from Dyno Particles. Columns of I.D. 4.6 mm, 8.0 mm and 16.0 mm and various lengths were slurry-packed in methanol (maximum pressure 150 bar) in our laboratory.

HPLC separation. All separations were performed at room temperature except for the Nucleosil separations with TFA–acetonitrile, which were carried out at 45°C. Detailed descriptions of the mobile phases and gradients used are given in the figure legends. Recoveries were calculated by comparing the area under the UV-curve after gradient elution of the specific column with that obtained after bypassing the column with 1.5-m PTFE capillary tubing.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a Pharmacia Phast Gel apparatus with 8–25% gradient gels. Electrophoresis and silver staining were performed as described by the manufacturer (Pharmacia Bulletin, Nos. 110 and 210).

Samples

Interleukin-1β, insulin (crystalline, porcine and highly purified human), human proinsulin and human growth hormone were obtained from Novo Nordisk. Other polypeptides were purchased from Sigma or Aldrich.

Pancreatic glands were obtained from the University Hospital, Copenhagen. From normal individuals classified as kidney donors, the pancreas was removed and frozen shortly after death. Only smaller parts of the pancreas were available from this category. From IDDM patients (insulin-dependent diabetics, age and diabetes duration unknown), the whole pancreas was removed after the ischaemia time (6–8 h according to the Danish death criteria). The pancreatic glands were kept frozen (*ca.* −30°C) after removal.

Before extraction, the pancreatic tissue was lyophilized and ground to a coarse powder. As 3 *M* acetic acid was found to be the optimum concentration for pancreas extraction (B. Hansen and S. Linde, unpublished results), the tissue was lyophilized before extraction in order to avoid any uncontrolled dilution of the acetic acid concentration in the added extraction medium. Up to 40 g of lyophilized pancreas powder were mixed with 200 ml of 3 *M* acetic acid, minced in a Warring Blender for *ca.* 1 min and extracted under magnetic stirring for 60 min. All operations were performed at 4°C. The extract was centrifuged at 4°C for 20 min in a Sorvall RC-5 cooled

centrifuge (25 000 *g*), the supernatant decanted and the tissue re-extracted twice under similar conditions.

The crude acetic acid extracts were analysed as such, or after lyophilization. Especially the diabetic extracts were difficult to lyophilize; the residue often had an oily appearance, probably owing to the presence of lipids in the crude extract.

Gel chromatography

Lyophilized acetic acid extracts were separated on a 90 × 2.5 cm I.D. Sephadex G-50 column eluted at 4°C with 3 *M* acetic acid at a flow-rate of *ca.* 20 ml/h. The column eluate was monitored continously at 280 nm (Uvicord II) and 15-min fractions were collected in an LKB 7000 Ultrorac fraction collector. A 200-mg amount of lyophilized sample was dissolved in *ca.* 25 ml of 3 *M* acetic acid and filtered [coarse filter-paper, followed by filtration on Millipore filters (5, 1.2, 0.8 and finally 0.45 μm)] before application.

Gel chromatography of 100 mg of crystalline porcine insulin, performed under similar conditions, resulted in the separation of the b-component [primarily containing the covalent insulin dimer (12 000 dalton) and proinsulin (9000 dalton)] and the c-component (containing insulin peptide and insulin-like material, MW 6000 dalton).

The components in the acetic acid extracts obtained after gel chromatography were collected into two fractions: peak I (the main peak, MW > 6000 dalton) and peak II (the smaller peak with elution volume greater than the main peak, MW *ca.* 6000 dalton). Peak I and II material was isolated from the column eluate by lyophilization.

Protein determination

The protein content in the extracts was measured using the Coomassie Brilliant Blue protein assay (Bio-Rad Labs.) with bovine serum albumin as a standard, as described by the manufacturer. Before analysis, the extracts were diluted to 0.1 *M* acetic acid.

Insulin determination

Insulin radioimmunoassays (RIAs) were carried out essentially as described [2] using a monoiodinated insulin tracer obtained from Novo-Nordisk.

Amino acid sequencing

Amino acid sequencing was performed using a gas-phase sequencer (Applied Biosystems 475) equipped with an on-line Model 120A PTH analyser and a Model 900A data module. The sequence data were compared with published amino acid sequences in a database [Genetics Computer Group, UVGCG NRBF Protein, version 6.0 (April 89)].

RESULTS

Interleukin-1β (IL-1β), a 17 400-dalton polypeptide with two free SH groups, is a "sticky" molecule which binds so strongly to several silica-based C_{18} and C_8 columns that the use of TFA–acetonitrile as the mobile phase was insufficient for elution and/or separation [3]. Only a C_4 column was found to be useful for TFA–acetonitrile-

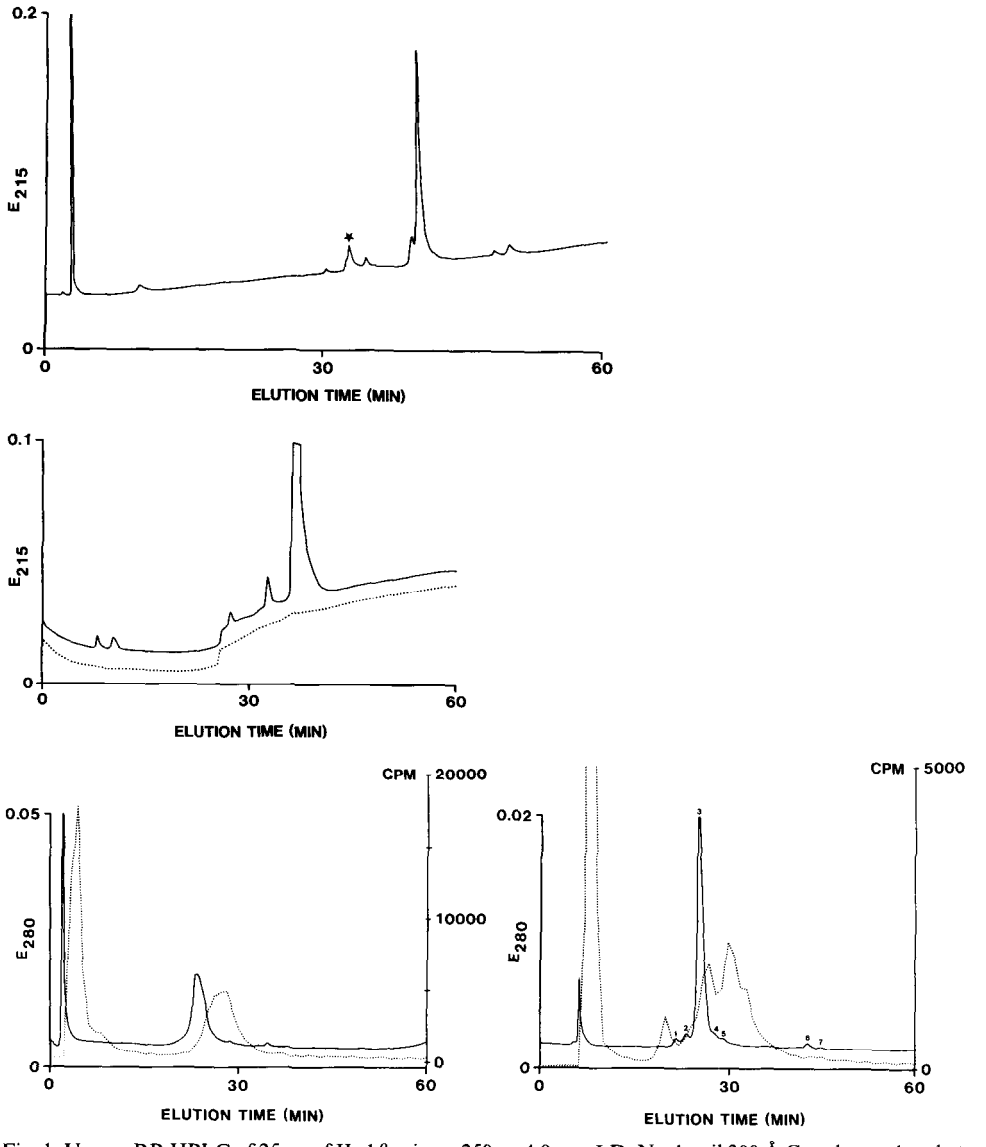

Fig. 1. Upper: RP-HPLC of 25 μg of IL-1β using a 250 × 4.0 mm I.D. Nucleosil 300 Å C_4 column eluted at 45°C with an acetonitrile gradient (10 → 50% linearly during 60 min) in 0.05% TFA. Flow-rate, 1.0 ml/min. The peak marked with an asterisk is a "ghost" peak. Middle: HPLC of 25 μg of IL-1β (solid curve) using a 100 × 7.5 mm I.D. Pentax PEC 102 hydroxyapatite column eluted with a linear phosphate gradient [0.01 *M* Na_2HPO_4–0.3 m*M* $CaCl_2$ (pH 6.8) → 0.4 *M* Na_2HPO_4–0.0075 m*M* $CaCl_2$ (pH 6.8) during 45 min followed by 15-min isocratic elution with the final buffer]. Flow-rate, 0.5 ml/min. Dotted curve, blind injection. Lower left: RP-HPLC of 50 μg of IL-1β (solid curve) and [^{125}I]IL-1β (dotted curve) using a 75 × 4.0 mm I.D. TSK Phenyl 5PW RP+ column eluted with 24% acetic acid–12% acetonitrile isocratically for 10 min, thereafter → 30% acetic acid–30% acetonitrile linearly during 35 min, and then to 40% acetic acid–60% acetonitrile linearly during 15 min. Flow-rate, 0.5 ml/min. Lower right: RP-HPLC of 25 μg of IL-1β (solid curve) and [^{125}I]IL-1β (dotted curve) using a 250 × 4.6 mm I.D. Dynosphere PD-102-RE column eluted with an acetic acid–acetonitrile gradient (27% acetic acid–21% acetonitrile → 32% acetic acid–36% acetonitrile linearly during 60 min). Flow-rate, 0.5 ml/min.

based RP-HPLC of this polypeptide (Fig. 1, upper). In addition, a fairly good separation was obtained when a hydroxyapatite column was eluted with a phosphate gradient (Fig. 1, middle), but in order to optimize further the separation between native IL-1β and closely related IL-1β-like impurities, a polymeric phenyl column (TSK Phenyl 5PW RP+) was eluted with an acetic acid–acetonitrile gradient (found to be useful for the separation of very hydrophobic SDS-solubilized membrane proteins from erythrocyte ghosts [1]). Under these conditions, purified recombinant-derived IL-1β was separated in a main component and a few minor constituents (Fig. 1, lower left, solid curve), but a better separation was obtained when another polymeric phenyl-based column, Dynosphere PD-102-RE, was eluted with the same mobile phase (Fig. 1, lower right, solid curve). A major component, constituing 90–95% of the sample material, was separated from six minor contaminants. The peak shape and separation capacity of this system were superior to those obtained using the above-mentioned C_{18}/C_8 and hydroxyapatite systems, and slightly better than that obtained using the silica-based C_4 columns eluted with TFA-acetonitrile (*cf.*, Fig. 1, upper).

For further evaluation of the selectivity of the two polymeric stationary phases eluted with acetic acid–acetonitrile a [^{125}I]IL-β preparation containing *ca.* 1 atom I/mol IL-1β was applied to the columns. Using the TSK Phenyl column [^{125}I]IL-1β was eluted as a single component, partially separated from native IL-1β (Fig. 1, lower left, dotted curve), whereas four major [^{125}I]IL-1β components were separated using the Dynosphere PD-102-RE column (Fig. 1, lower right, dotted curve).

As the separation of native IL-1β using the TFA–acetonitrile–C_4 system was comparable to that obtained on the Dynosphere column, [^{125}I]IL-1β was analysed under conditions similar to those used in Fig. 1, upper. However, the radioactivity was eluted as a single component with a similar retention time to native IL-1β [3]. Similar results were obtained when [^{125}I]IL-1β was analysed on the hydroxyapatite column [3]. The recoveries of IL-1β and iodinated IL-1β from the Dynosphere PD-102-RE column were found to be identical (*ca.* 80%).

In order to evaluate the potential harmful influence of acetic acid–acetonitrile on the biological activity of IL-1β, the column eluates containing components 1–7 shown in Fig. 1, lower right, were exchanged with RPMI 1640 and the content of IL-1β (ELISA assay) and the biological activities in the leukocyte activating factor (LAF) assay were determined as described [4]. The specific biological activity of the main component (marked "3") was found to be slightly higher than that of native IL-1β and those of the six minor components slightly reduced or similar to those in native IL-1β (Table I), indicating that the chromatographic procedure had no deleterious influence on the resulting specific biological activity. The specific biological activity of the main peaks isolated after hydroxyapatite chromatography (Fig. 1, middle) and also after RP-HPLC on Nucleosil C_4 eluted with TFA–acetonitrile (Fig. 1, upper) were determined in parallel, and found to be comparable to that of native IL-1β and IL-1β which had been incubated for 60 min in 0.085% TFA–80% acetonitrile (Table I).

For further characterization of the Dynosphere column, a number of commercially available peptides and proteins were eluted with three different mobile phases of acetic acid gradients in acetonitrile (Fig. 2, upper curve) in isopropanol (Fig. 2, middle curve) and in water (Fig. 2, lower curve). In the three experiments the column was

TABLE I

SPECIFIC BIOLOGICAL ACTIVITIES OF INDIVIDUAL FRACTIONS OBTAINED AFTER RP-HPLC OF PURIFIED IL-1β IN ACETIC ACID–ACETONITRILE (SEE FIG. 1, LOWER RIGHT), ON A HYDROXYAPATITE COLUMN (SEE FIG. 1, MIDDLE) OR ON A SILICA C_4 COLUMN ELUTED WITH TFA–ACETONITRILE (SEE FIG. 1, UPPER).

Before analysis in the ELISA and LAF assay (performed as described [4]), the solvents in the RP-column eluate were exchanged with RPMI 1640 on NAP-5 columns.

Fraction	Peak No.	Units/mg ($\times 10^{-8}$)
From Fig. 1, lower right	1	2.9
	2	3.5
	3	6.8
	4	3.6
	5	4.5
	6	4.6
	7	5.6
From Fig. 1, middle	Main peak	7.9
From Fig. 1, upper	Main peak	7.2
Native IL-1β		5.7
Native IL-1β desalted in RPMI		5.0
Native IL-1β after 60 min in 0.085% TFA–80% acetonitrile		4.6

eluted with linear 60-min gradients. Although it was observed that the efficiency gradually decreased when acetonitrile was replaced with isopropanol and acetic acid (most clearly seen in the chromatographic behaviour of β-lactoglobulin in the three mobile phases), it was also found that insulin could be eluted as a narrow peak with an acetic acid gradient, similarly to the behaviour of the much more hydrophobic human growth hormone (hGH, MW 22 000 dalton).

The elution of 2 mg of crystalline porcine insulin from a 16.0 mm I.D. Dynosphere PD-102-RE column using essentially the same three mobile phases in similar gradients is shown in Fig. 3, left. Under these conditions, the elution strength of acetic acid is lower than that of acetonitrile and isopropanol. The chromatograms are similar with respect to the peak shape of the main component (insulin peptide), but the insulin-like contaminants are better separated from the main component in the acetic acid gradient. The recovery of crystalline insulin was measured for the three mobile phases and found to be 86% (acetonitrile), 76% (isopropanol) and 85% (acetic acid in water).

Biosynthetic hGH analysed under similar conditions to crystalline insulin eluted as a single component in all three mobile phases (chromatograms not shown), and the recoveries were found to be 76% (acetonitrile), 85% (isopropanol) and 90% (acetic acid in water).

In order to investigate the separation capacity of the Dynosphere PD-102-RE column for complex biological samples, an acetic acid extract of a normal human pancreas (removed and frozen immediately after death) was analysed using the above three mobile phases. Pancreatic tissue was extracted as described under Experimental and, after centrifugation, the aqueous extract was isolated from residual tissue and a lipid layer. A 200-μl volume of this raw extract, containing *ca.* 3 mg of protein, was applied to a 180 × 16.0 mm I.D. Dynosphere column and eluted at 4.0 ml/min with a

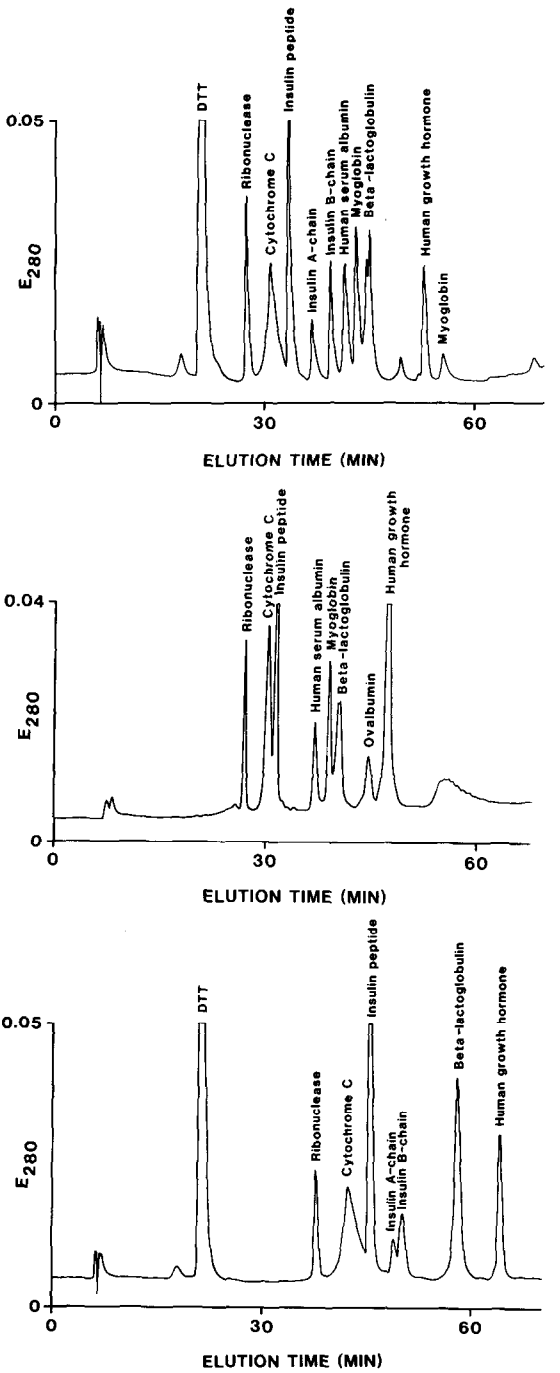

Fig. 2. Separation of a number of "standard proteins" using a 250 × 4.6 mm I.D. Dynosphere PD-102-RE column eluted with a linear gradient (25% acetic acid–15% acetonitrile → 40% acetic acid–60% acetonitrile during 60 min followed by 10 min isocratically at 40% acetic acid–60% acetonitrile (upper), 25% acetic acid–15% isopropanol → 40% acetic acid–60% isopropanol during 60 min followed by 10 min isocratically at 40% acetic acid–60% isopropanol (middle) or 37.5 → 90% acetic acid during 60 min followed by 10 min isocratically at 90% acetic acid). Sample amount, 20–50 μg; only the individual proteins indicated in the three panels were applied in each analysis. Flow-rate, 0.5 ml/min.

60-min linear gradient (Fig. 3, right). Apart from the already observed difference in elution strength between the three mobile phases, the overall elution patterns of this complex mixture were comparable in the three cases.

RP-HPLC of three pancreatic polypeptides, insulin, proinsulin and glucagon, eluted with an acetic acid gradient is shown in Fig. 4, left. All polypeptides were eluted as very narrow, symmetrical peaks, and the impurities in crystalline glucagon were very well resolved. Glucagon and insulin were eluted with the same retention time under these conditions, whereas monoiodinated glucagon was separated from the native glucagon (Fig. 4, right). The component that eluted *ca.* 15 min after glucagon is human serum albumin added to the [^{125}I]glucagon preparation.

To evaluate the usability of the acetic acid–Dynosphere system for the further

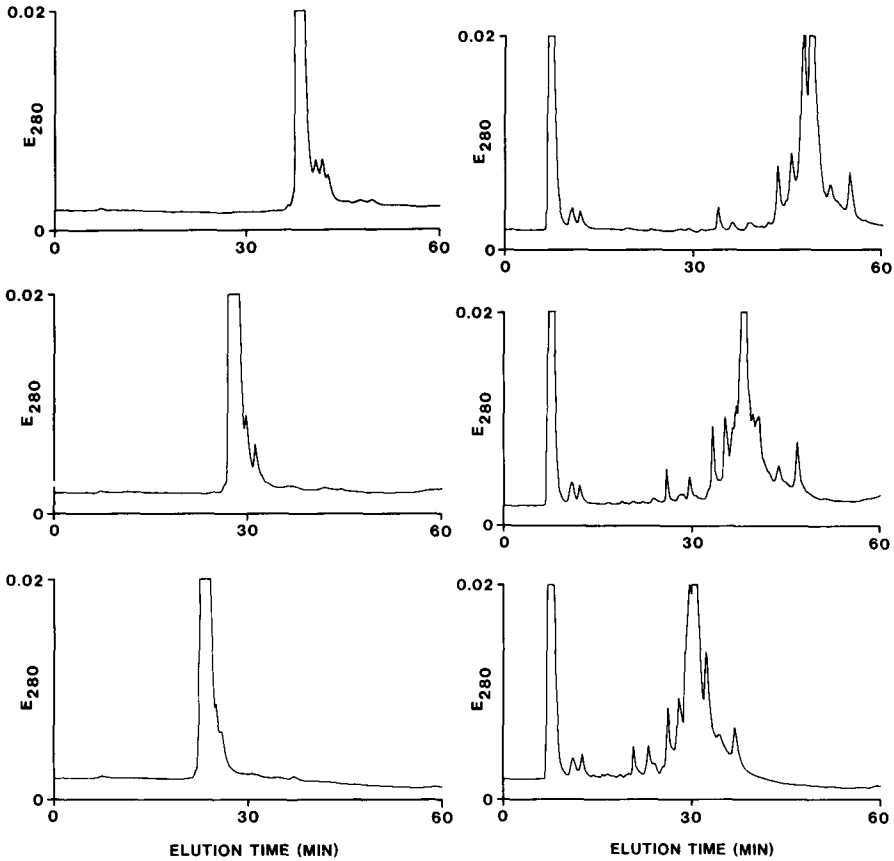

Fig. 3. Left: RP-HPLC of 2 mg of crystalline porcine insulin using a 180 × 16.0 mm I.D. Dynosphere column eluted with acetic acid in water, isopropanol or acetonitrile as organic modifiers. All gradients were linear during 60 min. Flow-rate, 4.0 ml/min. Upper curve: 37.5 acetic acid → 90% acetic acid. Middle curve: 25% acetic acid–15% acetonitrile → 40% acetic acid–60% acetonitrile. Lower curve: 25% acetic acid–15% isopropanol → 40% acetic acid–60% isopropanol. Right: separation of 200 µl of crude acetic acid extract of a normal human pancreas using a 180 × 16.0 mm I.D. Dynosphere PD-102-RE column eluted as described for the left panel.

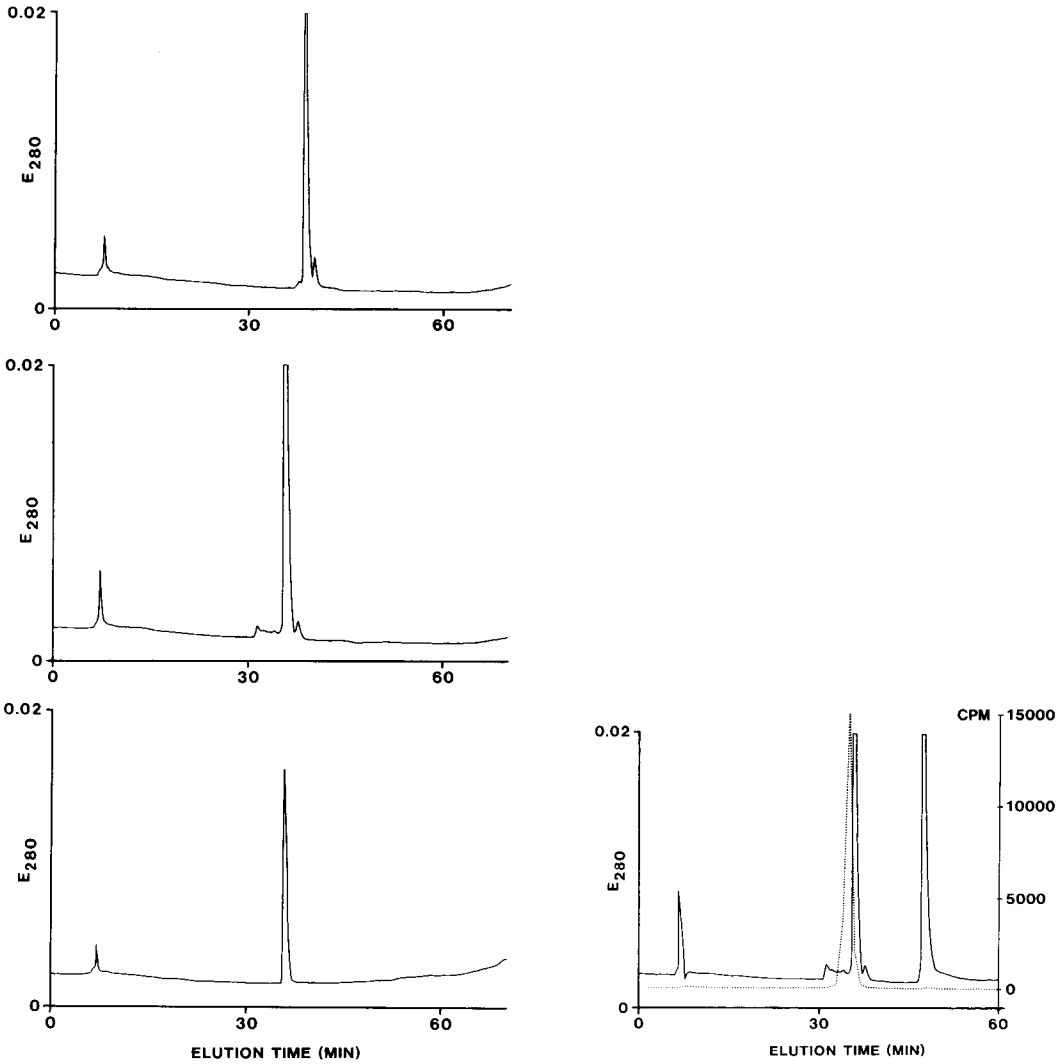

Fig. 4. Left: RP-HPLC of 10 μg of human proinsulin (upper curve), 15 μg of crystalline porcine glucagon (middle curve) and 6 μg of human insulin (lower curve) using a 280 × 4.6 mm Dynosphere PD-102-RE column eluted with a linear acetic acid gradient (37.5 acetic acid → 90% acetic acid) during 60 min. Flow-rate, 0.5 ml/min. Right: RP-HPLC of a mixture of 15 μg of crystalline porcine glucagon and *ca.* 35 000 cpm [^{125}I]glucagon. UV registration, solid curve; radioactivity, dotted curve. Stationary and mobile phases as in the left panel.

characterization of acetic acid extracts of normal and diabetic pancreatic glands, another normal and two diabetic pancreatic glands were extracted in 3 *M* acetic acid and the crude extracts were separated in a 37.5 → 90% acetic acid gradient (Fig. 5). The chromatograms of the extracts were found to be very different: the proteins extracted from the diabetic pancreata were eluted much earlier than those in the normal pancreas, indicating less hydrophobicity and/or lower molecular weight, and

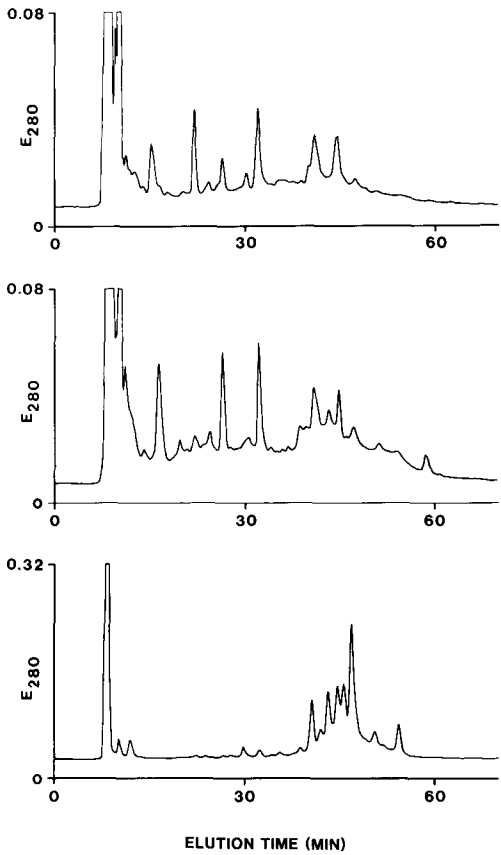

Fig. 5. RP-HPLC of 200 μl of crude actic acid pancreas extract from two human diabetics (upper and middle curves) and a normal human (lower curve) using a 280 × 4.6 mm I.D. Dynosphere column eluted with an acetic acid gradient (37.5 acetic acid → 90% acetic acid during 60 min) followed by 10 min isocratically at 90% acetic acid. Flow-rate, 0.5 ml/min.

the amount of extractable peptides/proteins in the crude extracts from the normal pancreatic glands was much higher than in those from diabetics (compare the UV scales).

RP-HPLC of peak I and II material (obtained as described under Experimental) using the Dynosphere–acetic acid system is shown in Fig. 6 (left, normal pancreas; right, diabetic pancreas). The separation of peak I materials from the diabetic pancreas was much less detailed than that of the instantly analysed crude extract (*cf.*, Fig. 5, upper and middle curves), whereas the peak II materials from the normal and the diabetic pancreata were both very well resolved.

In order to compare the above-mentioned separations of the pancreatic extracts (using acetic acid as mobile phase) with a commonly used RP-HPLC polypeptide separation system, peak I and II material were separated on a Nucleosil 300 Å C_4 column eluted with TFA–acetonitrile. The separation pattern obtained for peak I and II material from the normal pancreas (Fig. 7, upper left) and the diabetic pancreas

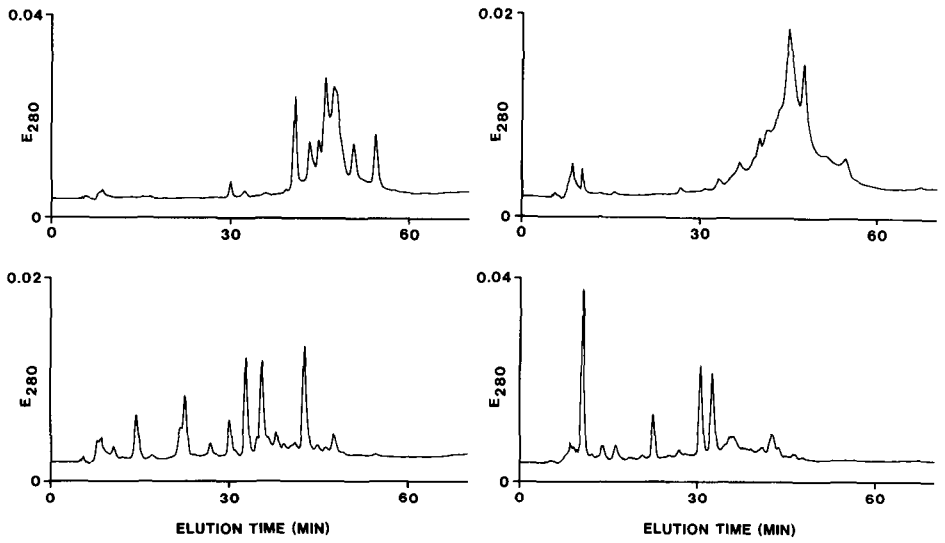

Fig. 6. Left: RP-HPLC of peak I (upper curve) and peak II material (lower curve) from normal human pancreas, redissolved in 3 *M* acetic acid to 1.0 mg/ml, centrifuged and filtered (0.45 μm). 200 μl were applied. Stationary and mobile phases as in Fig. 5. Flow-rate, 0.5 ml/min. Right: RP-HPLC of peak I material (upper curve) and peak II material (lower curve) from a diabetic human pancreas. 200 μl were applied. All conditions as in Fig. 5.

(Fig. 7, upper right) were more detailed than those obtained with the Dynosphere–acetic acid system (Fig. 6), but the distributions of components throughout the chromatograms in the two highly different RP systems were very similar both for the normal pancreatic extracts (Fig. 6, left, *versus* Fig. 7, upper left) and for the diabetic pancreatic extracts (Fig. 6, right, *versus* Fig. 7, upper right).

The UV absorption of the column eluate from the Nucleosil C_4–TFA–acetonitrile system was monitored at 215 nm, whereas the eluate from the Dynosphere–acetic acid system had to be monitored at a higher wavelength (280 nm). In order to compare the separations at a similar wavelength, peak I and II material from a diabetic pancreas was separated in the Nucleosil–TFA–acetonitrile system followed by detection at 280 nm (Fig. 7, lower). When these chromatograms are compared with Fig. 6, right, it can be seen that the separation patterns of the same sample in two different RP systems now appear to be very similar.

SDS-PAGE of the first, second and third crude acetic acid extracts of a normal pancreas, shown in Fig. 8, indicated a highly heterogeneous protein–peptide mixture with a molecular weight distribution in the total fractionation range (6000–300 000 dalton).

Finally, a series of crude extracts of recombinant-derived hGH-producing *Escherichia coli* were analysed. The crude bacterium pellet or disrupted bacteria were extracted in acetic acid and separated in the Dynosphere–acetic acid system (Fig. 9 A and B). In both instances, hGH was eluted in the late part of the chromatogram, well separated from all residual sample components. As can be seen from SDS-PAGE of

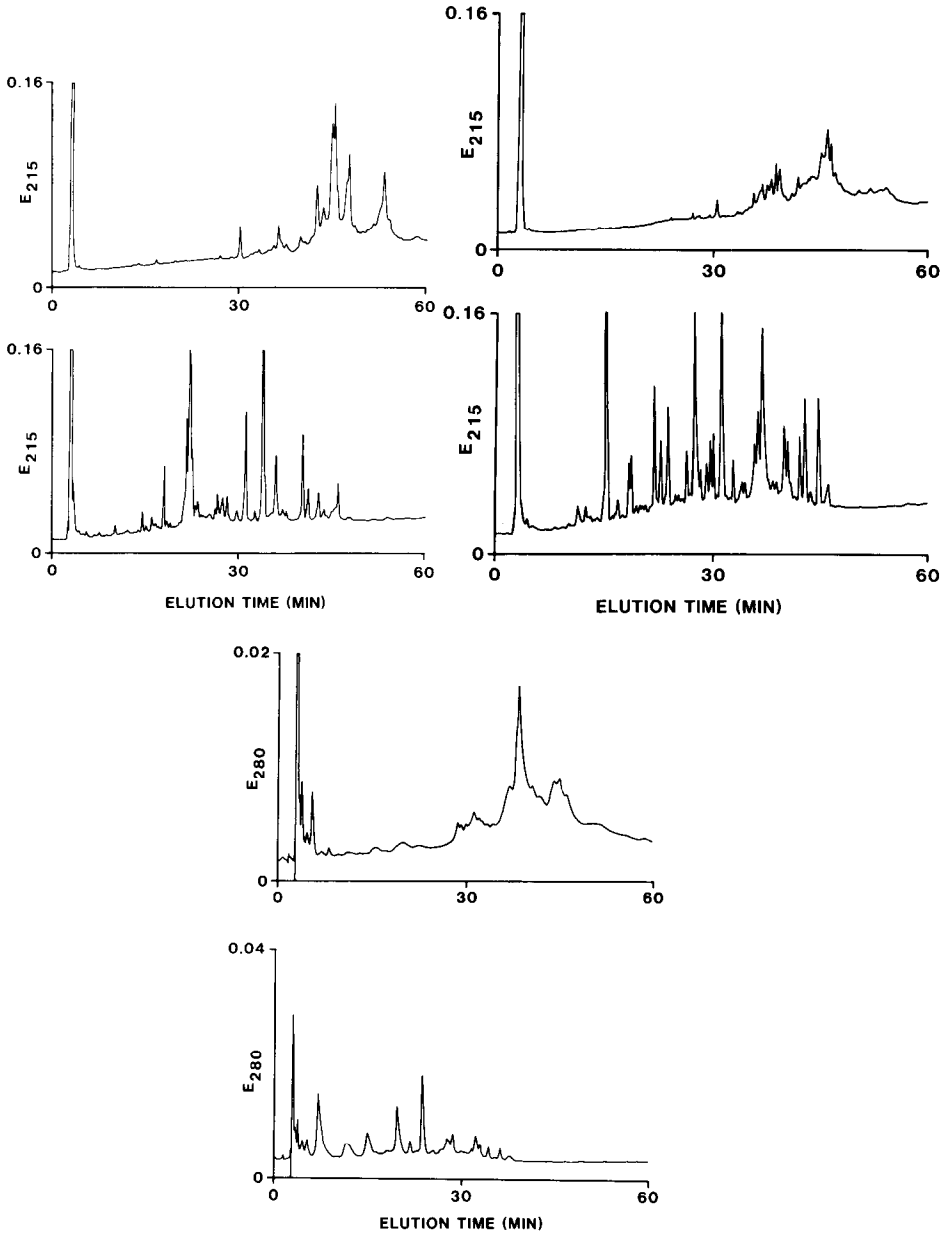

Fig. 7. Top: RP-HPLC of peak I material (upper curve) and peak II material (lower curve) from a normal human pancreas (left) and from a diabetic, human pancreas (right) using a 250 × 4.0 mm I.D. Nucleosil 300 Å C_4 column eluted at 45°C with an acetonitrile gradient (12 → 57% acetonitrile linearly during 60 min) in 0.1% TFA. 50 μl were applied in all analyses. Flow-rate, 1.0 ml/min. Bottom: RP-HPLC of peak I material (upper curve) and peak II material (lower curve) from a diabetic, human pancreas. 200 μl were applied. Stationary and mobile phases as for the top panels. Flow-rate, 1.0 ml/min. UV-registration, 280 nm.

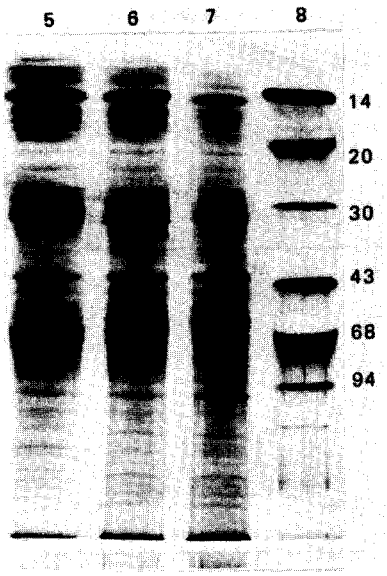

Fig. 8. SDS-PAGE of first, second and third acetic acid extracts of a normal, human pancreas (lanes 5, 6 and 7). Lane 8: molecular weight markers. The figures correspond to the MW in kilodalton.

the isolated hGH fractions, this single-step procedure resulted in a very efficient purification of the hGH preparation (Fig. 9C).

The acetic acid extract of the disrupted hGH *E. coli* bacteria was also analysed in the Nucleosil–TFA–acetonitrile system (Fig. 9D). The separation pattern was similar to that obtained with the Dynosphere–acetic acid system with respect to distribution of the components throughout the chromatogram, but is was also obvious that the peak capacity of the former system is higher.

DISCUSSION

Hydrophobic peptides and proteins may constitute a major problem in RP-HPLC owing to their high affinity to the stationary phase ligands (whether they are alkyl or phenyl groups), and conventional mobile phase additives (acetonitrile, alcohols) are often unable to break the hydrophobic bindings. Detergents, which may be useful for similar problems in the size exclusion of hydrophobic polypeptides, have hardly ever been reported as mobile phase additives in RP-HPLC, probably because they are almost irreversibly bound to the ligands, thereby totally changing the surface characteristics of the reversed stationary phase.

We have been able to elute a number of erythrocyte ghost membrane proteins with MW $>$ 100 000 dalton with good recovery from a phenyl column using a mobile phase based on a high content of acetic acid plus acetonitrile [1]. The reason for this surprising separation was probably the acetic acid in combination with the stationary phase phenyl groups, which normally binds peptides and proteins less strongly than C_8 and C_{18} columns.

We therefore chose the same stationary and mobile phase for the initial charac-

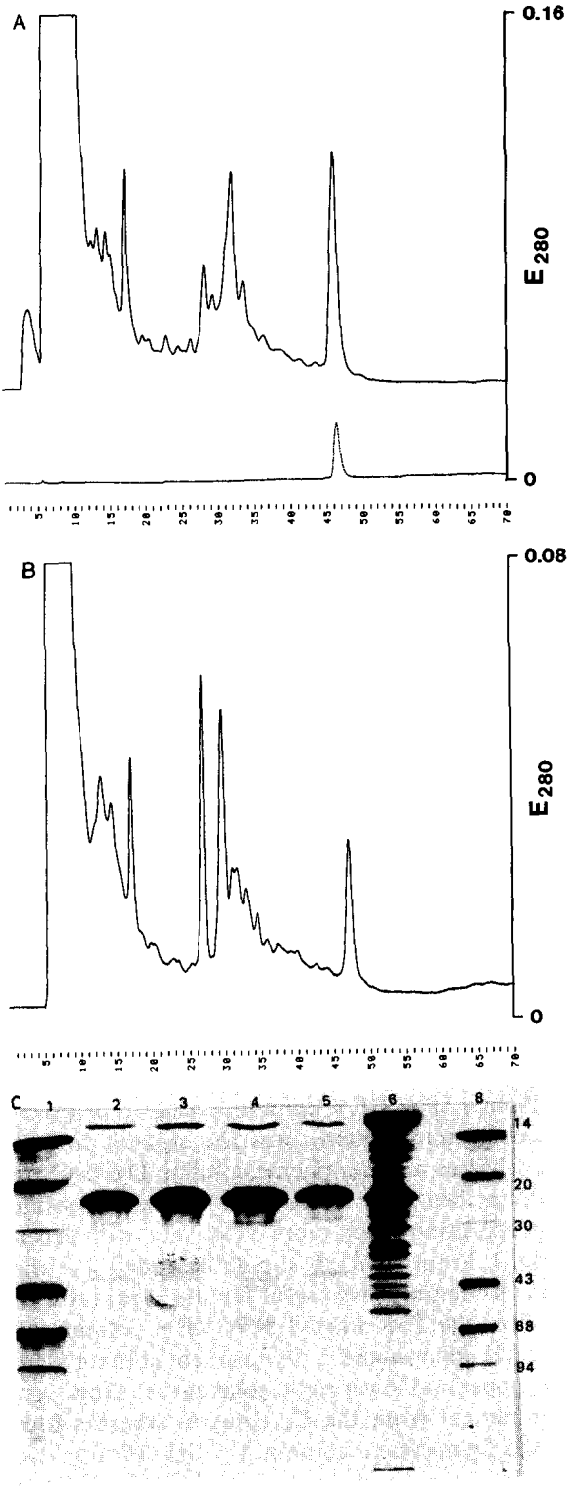

Fig. 9.

(Continued on p. 196)

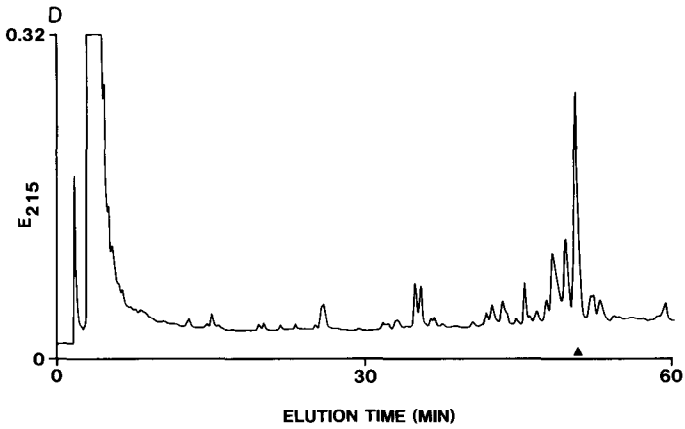

Fig. 9. (A) RP-HPLC of acetic acid extracts of disrupted hGH-producing *E. Coli*. A 0.75-ml bacteria residue pellet was extracted for 2.5 h at 4°C in 50% acetic acid. After centrifugation, the supernatant was diluted 1:1 with distilled water and 1800 μl were applied to a 150 × 8.0 mm I.D. Dynosphere PD-102-RE column. Elution was performed with a slightly concave 60-min gradient (37.5 → 90% acetic acid, Waters Assoc. M660 gradient controller, gradient No. 5) followed by 10 min isocratically at 90% acetic acid (upper curve). The lower curve shows the elution pattern of 50 μg of highly purified hGH under similar conditions. Flow-rate, 1.0 ml/min. The numbers on the abscissa are the elution times in minutes. (B) RP-HPLC of hGH-producing *E. Coli* extracted directly with acetic acid. A 0.5-ml bacteria pellet was extracted with 2.5 ml of glacial acetic acid at 4°C for 2.5 h. After centrifugation, the supernatant was diluted with distilled water (1:1.5), and 1000 μl were applied to a 150 × 8.0 mm I.D. Dynosphere PD-102-RE column eluted as described for (A). (C) SDS-PAGE of an acetic acid extract of disrupted hGH-producing *E. coli* (lane 6) and of hGH isolated after four individual RP-HPLC separations of this acetic acid extracts using the Dynosphere column under the conditions described for (A) (lanes 2–5, respectively). Lanes 1 and 8: molecular weight markers. The figures correspond to the MW in kilodalton. (D) RP-HPLC of an acetic acid extract of disrupted hGH-producing *E. Coli* using a 250 × 4.0 mm I.D. Nucleosil 300 Å C_4 column eluted with an acetonitrile gradient (25% → 50% acetonitrile linearly during 60 min) in 0.1% TFA. Flow-rate, 1.0 ml/min. The elution volume of biosynthetic human growth hormone is marked with a filled triangle.

terization of IL-1β, which is hydrophobic, but not to such an extent as membrane proteins; we found that the molecule hardly could be eluted from a C_8 column and that it bound irreversibly to a C_{18} column [3] whereas TFA–acetonitrile was well suited for the elution of IL-1β from a C_4 column (Fig. 1, upper). Similar observations have been published elsewhere [5].

It was interesting that the TSK Phenyl 5PW RP+ column, found to be excellent for separating erythrocyte ghost membrane proteins, was less suitable for the RP-HPLC of IL-1β (Fig. 1, lower left), whereas another phenyl column, Dynosphere PD-102-RE (based on polymerized divinylbenzene) eluted with the same mobile phase was able to separate the IL-1β preparation much more effectively, with excellent peak shape and good recovery (Fig. 1, lower right). It should be added that the Dynosphere column was found to be considerably less useful for the separation of erythrocyte ghost membrane proteins than the TSK Phenyl 5PW RP+ column [6]. The separations obtained using a silica C_4 column and a Dynosphere column were comparable in the case of IL-1β (Fig. 1, upper and lower right, solid curve). However, [^{125}I]IL-1β was eluted as a single component from the C_4 column, whereas four major fractions were separated using the Dynosphere column. [^{125}I]IL-1β was also

eluted as a single component from the TSK Phenyl column, illustrating the very different selectivities of the two polymeric phenyl columns (Fig. 1, lower panels, dotted curves) and the C_4 silica column.

Interestingly, the combined effects of acetic acid and acetonitrile and the binding to the reversed stationary phase had no effect on the specific biological activity of the purified IL-1β, which was found (within the experimental variations in the LAF assay) to be equal to that of IL-1β before RP-HPLC (Table I). Further, RP-HPLC in TFA–acetonitrile (Nucleosil C_4) was also without effect on the resulting specific biological activity, indicating that although IL-1β is a reactive molecule (two free SH groups), it is resistant towards the influence of the harsh mobile phases and the strong binding to the stationary phases, conditions known to be destructive for several other biological active polypeptides [7].

A hydroxyapatite column eluted with a phosphate gradient, a non-RP technique, was able to resolve the IL-1β preparation fairly well, although some tailing was observed for the main fraction (Fig. 1, middle). The specific biological activity of this main fraction was found to be equal to that of native IL-1β (Table I), indicating that hydroxyapatite chromatography should always be considered as a potential alternative for the RP-HPLC of peptides and proteins.

From the characterizations of the Dynosphere column with "standard proteins", insulin and raw pancreas acetic acid extract, it should be noted that in general, the mobile phase based solely on acetic acid was less efficient than those with acetonitrile and isopropanol (Fig. 2). It was most clearly demonstrated in the elution pattern of β-lactoglobulin: the separation of the two chains was distinct with acetonitrile (Fig. 2, upper), indicated with isopropanol (middle) but absent with acetic acid (lower).

However, the peak shape and recovery of hGH and insulin were ideal after elution with acetic acid alone (Fig. 2, lower, and Fig. 3, left). It is especially worth noting that the recovery of the hydrophobic 22 000-dalton hGH in an acetic acid gradient in water was 90%. Glucagon, glucagon-related impurities and monoiodinated glucagon were also well separated in the acetic acid gradient (Fig. 4), whereas glucagon and insulin peptide, in contrast to several acetonitrile-based RP-HPLC systems [8], were found to coelute.

As the separation of the complex pancreatic extract resulted in very comparable chromatograms in all three mobile phases (Fig. 3, right), we concluded that an acetic acid gradient could be used for further characterizing the extracts of the normal and the diabetic human pancreas. The fact that the diabetic pancreas contained less extractable peptides/proteins with considerably lower hydrophobicity and/or molecular weight than the normal pancreas (Fig. 5) may be explained by the fact that the normal pancreatic glands were "fresh" (obtained from persons exposed to sudden death, classified as kidney donors), whereas the diabetic pancreatic glands were removed after the ischaemia time (6–8 h according to the Danish death criteria). The pancreas is loaded with digestive enzymes, and proteolytic cleavage of peptide bonds in this period is very likely to take place.

After Sephadex G-50 chromatography of normal pancreatic extracts in 3 *M* acetic acid, the amount of material with MW $>$ 6000 dalton (95-98%) was substantially higher than the amount of peptides with MW $\leqslant$ 6000 dalton (1–2%) [6], indicating that the endocrine pancreas constitutes 1–2% of the total weight of the

pancreas, and that the major digestive enzymes constitutes *ca.* 75% of the total proteins present in the exocrine pancreas.

The fact that hGH was eluted very late in the chromatogram when the Dynosphere column was eluted with an acetic acid gradient in water, in combination with the high recovery for hGH with this mobile phase, makes the system very useful for purification of the crude acetic acid extracts of *E. coli* with the inserted hGH gene [9] (Fig. 9A and B). hGH was very well separated from the majority of co-extracted impurities, and only minor residual contaminants were detected, even when the SDS-PAGE was overloaded (Fig. 9). The fact that the extraction medium (similar to the acetic acid extracts of pancreatic glands) may be applied, directly or after dilution with water, to the RP column, makes this procedure very attractive for preparative purposes.

When the above-mentioned samples (IL-1β, extracts of human diabetic and normal pancreata after Sephadex G-50 chromatography and crude hGH *E. coli* extracts) were analysed using a Nucleosil 300 Å C_4 column eluted with TFA–acetonitrile (Figs. 1, 7 and 9), it is striking that the separation patterns for the two highly different RP systems were very similar in the overall distribution of the separated components. It is normally considered that the π-electrons in the aromatic ring lead to binding characteristics different from those obtained with alkyl groups, but in the systems used in this study, the chromatograms were found to be comparable, especially when the column eluates were monitored at similar wavelengths (Fig. 6, right, and Fig. 7, lower). However, for further studies of similarities and differences, the Dynosphere should be eluted with TFA–acetonitrile and the chromatograms compared with those obtained with acetic acid gradients. The opposite comparison (Nucleosil C_4 eluted with 37.5–90% acetic acid) is hardly realistic owing to potential solubility problems with the silica-based stationary phase.

The reason for the elution strength of acetic acid in this RP situation is not obvious. The use of high concentration of formic acid has been reported for the RP-HPLC of defatted egg yolk proteins, but only present in buffer A [72% formic acid, buffer B being isopropanol–acetonitrile (2:1)] [10]. Formic acid (60%) has been used as a mobile phase additive for the RP-HPLC of water-insoluble polio virus proteins [11] and bovine serum albumin derivatives and other hydrophobic proteins [12], but in these experiments the elution was performed with 10–30% isopropanol in formic acid (60% formic acid being present in both buffer A and buffer B). Formic acid itself, in concentrations up to 100%, eluted only very few proteins, and it was reported that replacing formic acid with acetic acid was unsuccessful, leading to non-ideal peak shapes and reduces recoveries [11]. The separation of β-lactoglobulin, eluted as two components with very good separation, indicates that the separation capacity of the formic acid–isopropanol system is different from that of the Dynosphere–acetic acid system described here, but equal to that obtained when the Dynosphere was eluted with an acetonitrile-containing mobile phase (Fig. 2, upper). The major reason for the differences between the formic acid system and the acetic acid system is probably the use of a C_8 column in the formic acid–isopropanol system [11,12] *versus* a phenyl column in this work. We eluted the Dynosphere column with a formic acid gradient (20 → 90% formic acid), but recovered virtually none of the proteins and peptides separated in Fig. 2, and the baseline was extremely unstable [6].

In conclusion, we have developed a reversed-phase system which allows sep-

aration of a number of individual peptides/proteins and complicated crude acetic acid extracts of various biological tissues. The separation capacity, peak shapes and recoveries were frequently excellent, and the elution could be be performed with acetic acid alone, without the commonly used organic modifiers. Further characterization of extracts of the normal and diabetic human pancreas will be published separately [13].

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