*Journal of Chromatography*, 542 (1991) 65–81
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 016

# Use of polymeric reversed-phase columns for the characterization of polypeptides extracted from human pancreata

# I. Effect of the mobile phase

BENNY S. WELINDER* and SUSANNE LINDE
*Hagedorn Research Laboratory, 6 Niels Steensensvej, DK-2820 Gentofte (Denmark)*
(First received August 20th, 1990; revised manuscript received November 23rd, 1990)

## ABSTRACT

The high-performance liquid chromatographic (HPLC) behaviour of two different styrene–divinylbenzene-based reversed-phase (RP) columns was evaluated using crude acetic acid extracts from normal and diabetic human pancreata as samples. Acetic acid gradients in water and acetonitrile gradients in triethylammonium phosphate (TEAP) and trifluoroacetic acid (TFA) were used as mobile phases, and comparisons were made with a silica-based $C_4$ column. When two different polymeric RP columns were eluted with acetic acid gradients in water, surprisingly similar HPLC profiles of the pancreatic extracts were obtained. Elution of the polymer-based columns with acetonitrile gradients in TFA or TEAP resulted in changes in the polypeptide selectivity of these columns, in parallel with that of a silica-based $C_4$ column eluted under similar conditions, indicating the general usability of polymeric columns for RP-HPLC of peptides and proteins. The pronounced difference in composition between normal and diabetic samples, which also was demonstrated after size-exclusion chromatography (SEC) on a silica-based and an agarose-based high-performance SEC column, was found to be related to the different ischaemia times for the two types of pancreata.

## INTRODUCTION

We have recently demonstrated that several polypeptides, including insulin and growth hormone, could be eluted from a divinylbenzene-based polymeric reversed-phase (RP) column eluted with an acetic acid gradient in water. The peak shapes were excellent, and the recoveries were comparable to those obtained after elution with acetic acid-based mobile phases containing "classical" organic modifiers (*i.e.*, acetonitrile or 2-propanol) [1]. This stationary phase–mobile phase combination was also found to be very useful for the RP high-performance liquid chromatographic (HPLC) characterization of crude acetic acid extracts of normal and diabetic human pancreas. Almost identical UV profiles were obtained for acetic acid gradients in acetonitrile, 2-propanol or water, and the elution patterns after RP-HPLC utilizing acetic acid gradients in water were found to be similar to those obtained when a silica-based $C_4$

0021-9673/91/$03.50 

column was eluted with acetonitrile gradients in trifluoroacetic acid (TFA) [2].

The elution patterns from the normal and the diabetic human pancreas were very different. Considerably smaller amounts of polypeptides could be extracted from the diabetic than from the normal pancreas, and the polypeptides detected after RP-HPLC were shifted significantly towards lower molecular weight and/or hydrophobicity. This finding might reflect that the normal pancreata were removed and frozen shortly after clinical death whereas the diabetic glands were taken out after more than 6 h ischaemia time (according to the Danish death criteria), leaving sufficient time for the digestive enzymes in the exocrine pancreas to carry into effect a pronounced proteolytic degradation.

In order to explain this point, we analysed the acetic acid-extractable polypeptides obtained from a number of diabetic and normal human pancreatic glands with similar ischaemia times, and the effect on "fresh", normal pancreata of a 6-h time period at room temperature before extraction. Protein determinations and insulin analyses [radioimmunoassay (RIA)] of the extracts were performed as well.

The performance of silica-based alkyl RP columns in polypeptide analyses is highly dependent on the actual mobile phase, especially the buffer components. Further, columns supposed to be identical (*e.g.*, "end-capped" $C_{18}$ columns from different manufacturers, or from the same manufacturer but from different batches) may behave differently after elution with identical mobile phases [3–5].

As the potential value of polymeric columns for RP-HPLC of polypeptides remains largely untested [3], we performed the analyses with acetic acid gradients in water on two different polymeric RP columns (based on polymerized divinylbenzene or styrene). With acetic acid extracts of a normal and a diabetic human pancreas as model samples, one of the polymer columns was further eluted with acetonitrile gradients in TFA and triethylammonium phosphate (TEAP), and the results were compared with those of similar separations performed on a silica-based $C_4$ column.

## EXPERIMENTAL

### *Apparatus*

Commercially available HPLC equipment and columns were used throughout: pumps, Waters Assoc. M510 and M6000A, Gynkotek 300C; sample injectors, U6K, WISP 710A and B (Waters Assoc.), 7125 (Rheodyne); UV detectors, Linear UVIS 200, Hitachi L4200; integrator, Hitachi L2500; and gradient controllers, Waters Assoc. M660, Gynkotek 250B and 480.

Asahipak ODP-50 (150 × 4.6 mm I.D.), C8P-50 and C4P-50 (250 × 4.6 mm I.D.), Chrompack 8P 300 RP (150 × 4.6 mm I.D.), 300 Å Nucleosil $C_4$ (5 $\mu$m, 250 × 4.0 mm I.D.), TSK Phenyl 5PW RP+ (75 × 4.6 mm I.D.), Tosoh octadecyl 4PW (150 × 4.6 mm I.D.) and Tosoh octadecyl NPR (35 × 4.6 mm I.D.) columns were obtained prepacked. Dynospheres PD-102-RE was obtained as a 10-$\mu$m packing material from Dyno Particles; 250 and 30 × 4.6 mm I.D. columns were slurry-packed in methanol in our laboratory (maximum pressure 150 bar). Zorbax Bio Series GF-250 and 450 columns (250 × 9.6 mm I.D.) and a 300 × 10.0 mm I.D. Superose 12 column were obtained from DuPont and Pharmacia, respectively.

*Chemicals*

Acetonitrile was obtained form Rathburn (HPLC grade S), acetic acid (analytical-reagent grade) from Merck and TFA (sequential grade) from Applied Biosystems. All other chemicals were of analytical-reagent, sequential or similar purity. Water was drawn from a Millipore Milli-Q plant. The mobile phases were Millipore-filtered (0.45 $\mu$m) and degassed (vacuum/ultrasound) before use. During chromatography, the mobile phases were degassed continuously by passage through an ERMA ERC 3310 or a Shodex KT-35S degasser.

*HPLC separations*

All separations were performed at room temperature. Detailed descriptions of the stationary and mobile phases used are given in the figure legends.

*Polypeptide standards*

Highly purified human insulin and human proinsulin were obtained from Novo-Nordisk and crystalline porcine glucagon (same amino acid sequence as human glucagon) from Sigma.

*Samples*

Pancreatic glands were obtained from the University Hospital, Copenhagen. From normal subjects classified as kidney donors, the pancreas was removed and frozen shortly after death. Only non-identified parts (40–140 g frozen weight, 10–37 g after lyophilization) of these pancreata were available for this study. A number of normal and insulin-dependent diabetic human glands [obtained in their entirety, age and duration of insulin-dependent diabetes mellitus (IDDM) unknown] were obtained after 6–8 h ischaemia time.

Sample preparation was performed essentially as described [2]. Briefly, the pancreas was lyophilized, minced and extracted with 3 *M* acetic acid at 4°C. The acetic acid extract was analysed as such, or after lyophilization. Redissolved lyophilized pancreatic extracts were subjected to gel chromatography (Sephadex G-50 eluted with 3 *M* acetic acid at 4°C), and two major fractions were obtained: peak I [molecular weight (MW) > 6000 dalton] containing the major pancreatic digestion enzymes, albumin and globulins and peak II (MW $\leqslant$ 6000 dalton, salts being excluded). Peak I and II materials were isolated from the column eluate by lyophilization.

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

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

*Protein determinations*

The protein content in the extracts was measured in the BCA protein assay (Pierce) with bovine serum albumin as standard. Before analysis, the extracts were diluted to 0.1 *M* acetic acid.

*Insulin determinations*

In order to avoid unspecific binding of insulin, the pancreatic extracts were diluted 1:10 with 6 *M* urea and left for 1 h in this solvent before further dilution.

Insulin RIA was performed essentially as described [6]. Monoiodinated insulin tracer was obtained from Novo-Nordisk.

## RESULTS

The RP-HPLC separations of raw acetic acid extracts of a normal and a diabetic human pancreas using two different polymeric columns (Dynospheres PD-102-RE and Chrompack 8P 300RP) are shown in Fig. 1. The columns were eluted with 60-min linear acetic acid gradients in water (from 34 to 90% acetic acid), and the elution patterns were surprisingly similar. However, whereas insulin and glucagon coeluted from the Dynospheres column, they were separated on the Chrompack column.

We have recently shown that the elution patterns of extractable polypeptides from diabetic pancreata were very different from those from normal subjects, and

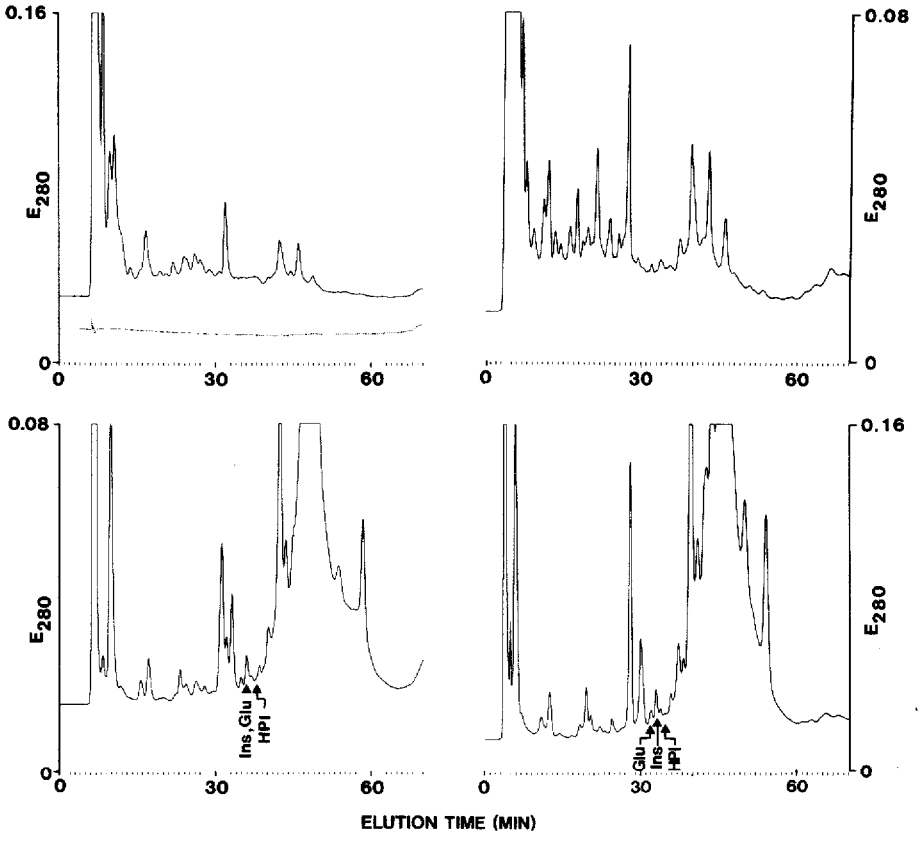


Fig. 1. RP-HPLC of 50 μl of acetic acid extract of a normal human pancreas (H93, bottom) and a diabetic human pancreas (H56, top) using a 250 × 4.6 mm I.D. Dynospheres PD-102-RE column (left) or a 150 × 4.6 mm I.D. Chrompack 8P 300 RP column (right) eluted with an acetic acid gradient (from 34% to 90% acetic acid linearly for 60 min, followed by 10 min isocratically at 90% acetic acid) at 0.5 ml/min. The elution positions of authentic human insulin (Ins), proinsulin (HPI) and glucagon (Glu), obtained after chromatography of the extracts spiked with the corresponding hormones, are marked.

that greater individual variations were found between the compositions of extracts from a few individual diabetic than from a few normal pancreatic glands [2]. We therefore extracted five different diabetic glands and analysed the extractable polypeptides on both polymeric RP columns. Two representative extracts are shown in Fig. 2. When these UV profiles are compared with those of a normal extract (lower curves in Fig. 1), it is evident that the dominant sample constituents in the normal extract (eluted in the last third of the chromatograms) are virtually absent from all diabetic extracts. Although similar in the outline, the elution patterns for the diabetic glands displayed a number of minor differences, and these differences are clearly and identically observed in the chromatograms from both polymeric RP columns.

The molecular size distribution in lyophilized acetic acid extracts from three normal and three diabetic pancreata were analysed on two different high-performance size-exclusion chromatography (HPSEC) columns. Typical chromatograms are shown in Fig. 3 for silica-based Zorbax GF-250 and GF-450 columns in series (right) and an agarose-based Superose 12 column (left). Although the distributions of sample components separated on the two different HPSEC columns are different, it is clearly seen that sample components with higher molecular weights were present in extracts form normal pancreata (Fig. 3, bottom) than in the extracts of diabetic glands (Fig. 3, top).

As the major difference between the handling of the normal and the diabetic glands was the ischaemia period, parts of three individual normal pancreata were thawed and kept at room temperature for 6 h before lyophilization and extraction. Two typical chromatograms obtained on the Dynospheres column are shown in Fig. 4 (top and middle). In spite of individual differences in the total amounts of extracted and eluted polypeptides, the overall distributions of sample components from the two pancreata are comparable to that of an instantly removed and frozen normal pancreas (compare with Fig. 1). Strikingly similar chromatograms were obtained for the same samples using the Chrompack column (data not shown).

Whole normal human pancreatic glands were obtained after similar ischaemia times as for the diabetic glands, and three of them were extracted and analysed as described above. The elution pattern of a single of these "normal" glands (Fig. 4, bottom) was different from that of the fresh normal gland shown in Fig. 1 and the fresh normal glands incubated for 6 h at room temperature (Fig. 4, top and middle): the UV profile was totally shifted towards that of a diabetic gland (as shown in Fig. 2) with almost total disappearance of the dominant sample components eluted in the last part of the gradient.

The protein and insulin contents in acetic acid extracts of normal glands with various time periods between removal, freezing and lyophilization/extraction are shown in Table I. As only non-identified parts of fresh normal glands were available, and as the distribution of hormone-producing cells is uneven throughout the gland [7–15], a variable insulin content in these extracts would be expected. In order to overcome this uncertain factor, a single part of a normal human pancreas was thawed, cut into several smaller pieces and mixed. Half of the pieces were lyophilized immediately and the other half were left at room temperature for 6 h before lyophilization. After extraction, the protein contents in the extracts from the two halves were found to be comparable (175 mg per g pancreas for the fresh half and 158 mg per g pancreas for the half incubated for 6 h at 22°C), whereas the insulin content was

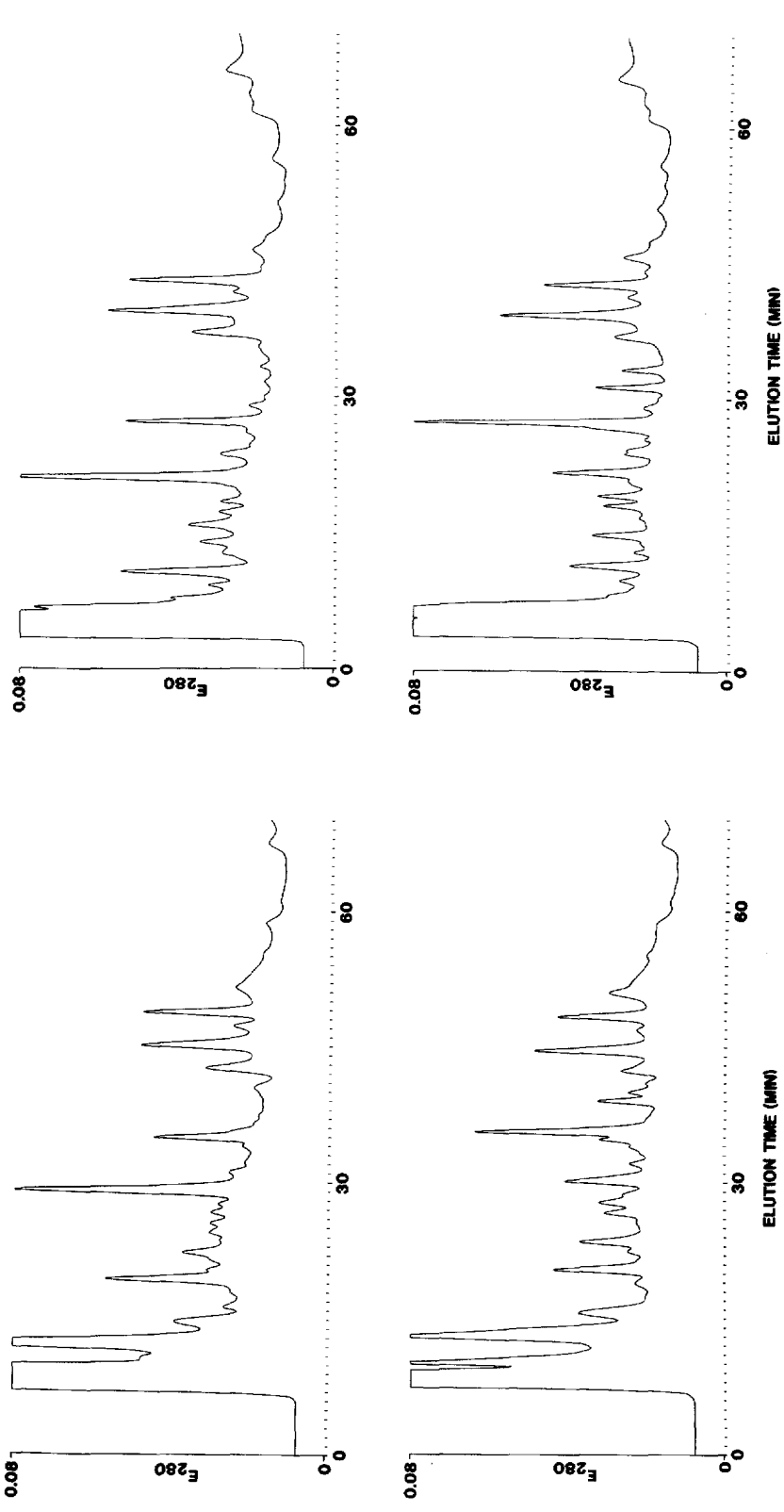


Fig. 2. RP-HPLC of 200 $\mu$l of acetic acid extract from two individual diabetic human pancreata using a 280 × 4.6 mm I.D. Dynospheres PD-102-RE column (top) or a 150 × 4.6 mm I.D. Chrompack 8P 300 RP column (bottom) eluted essentially as described in Fig. 1. The order of the two individual diabetic pancreatic glands (left, diabetic pancreas A; right, diabetic pancreas B) is identical in both the top and bottom panels.

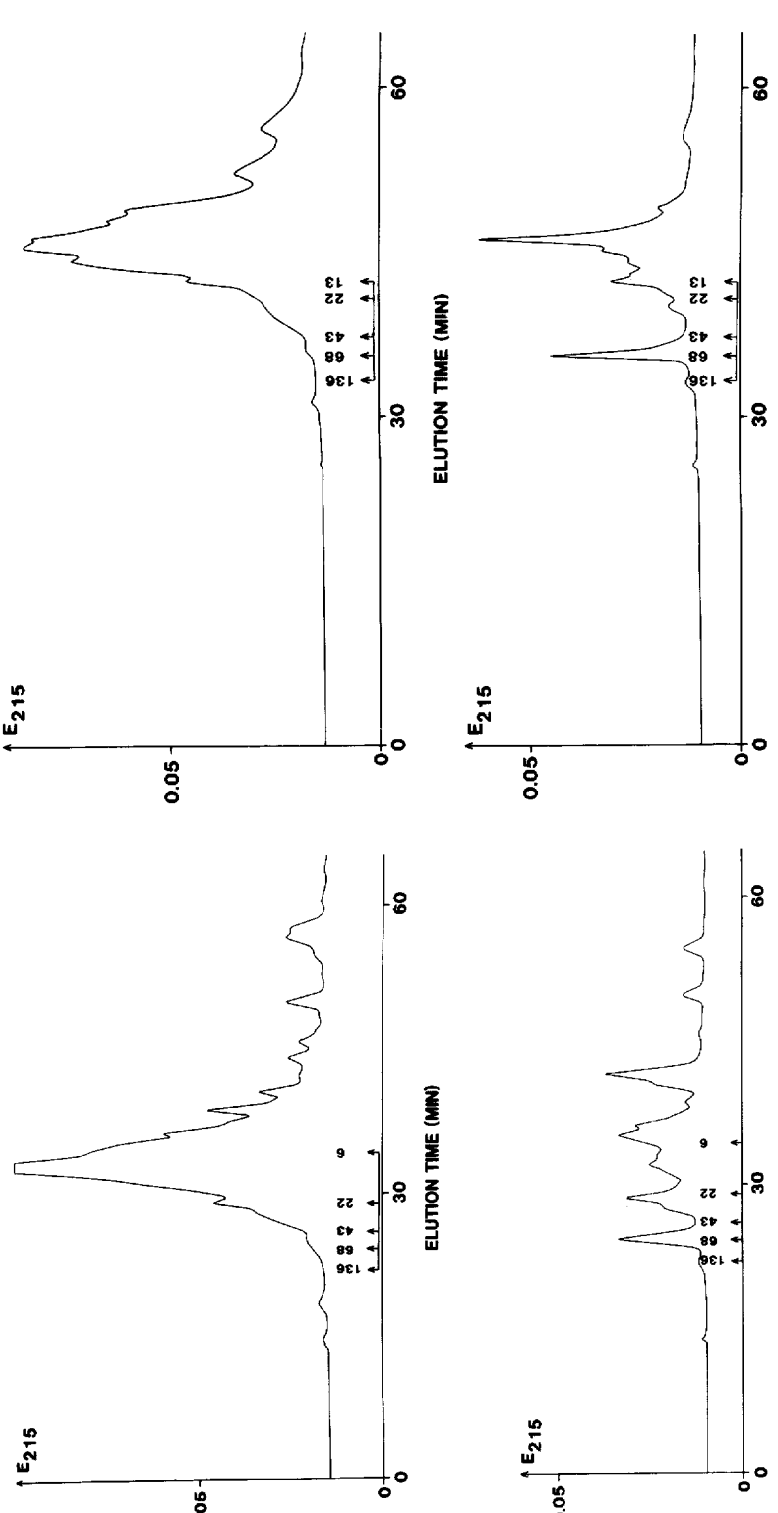


Fig. 3. HPSEC of lyophilized acetic acid extract from a diabetic (top) and a normal human pancreas (bottom) using a 300 × 10.0 mm I.D. Superose 12 column (left) or 250 × 9.4 mm I.D. Zorbax GF-250 and GF-450 columns connected in series (right). The columns were eluted at 0.5 ml/min with 0.2 *M* $Na_2HPO_4$ (pH 7.0). The lyophilized crude extracts were redissolved in the mobile phase to 1 mg/ml and 100 $\mu$l were applied to both columns. The elution positions of HSA dimer (136 000 dalton), HSA (68 000 dalton), ovalbumin (43 000 dalton), growth hormone (22 000 dalton), ribonuclease (13 000 dalton) and insulin (6000 dalton) are marked.

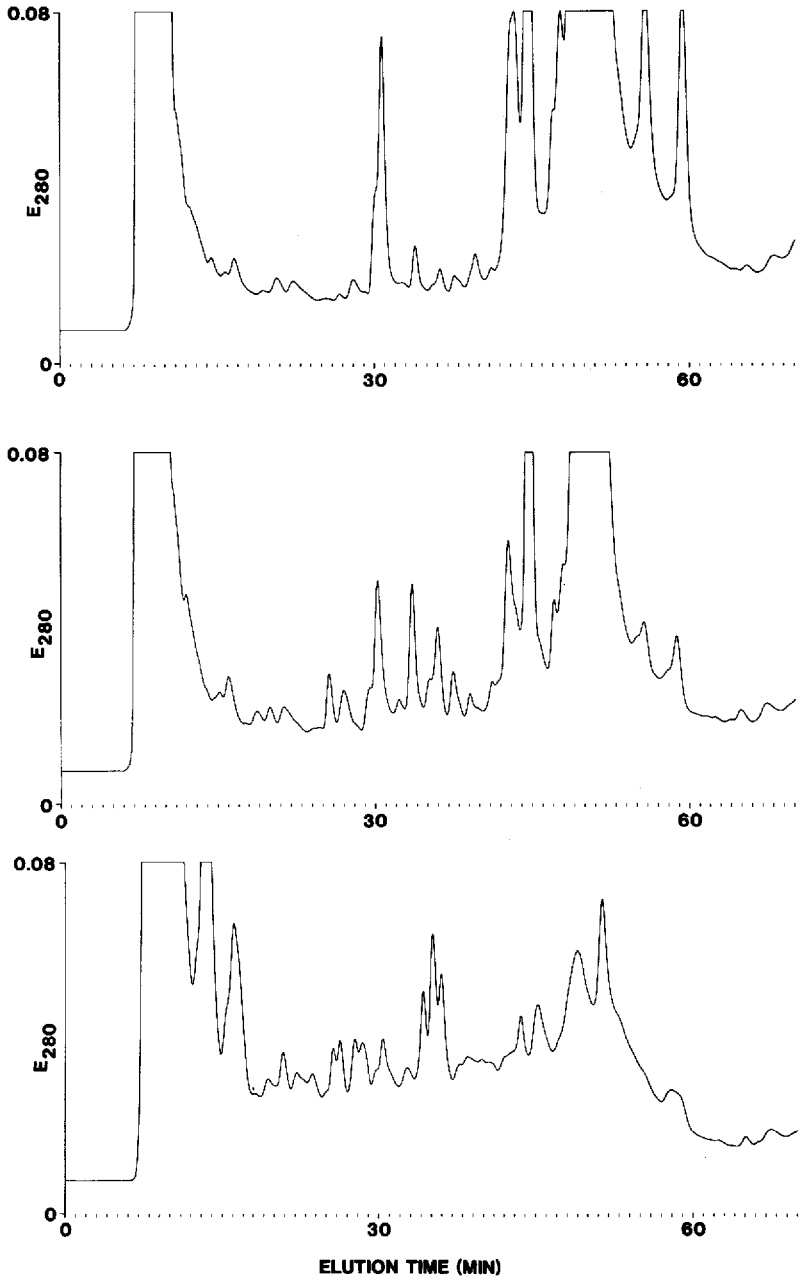


Fig. 4. Top and middle: RP-HPLC of 200 $\mu$l of acetic acid extract from two individual normal human pancreatic glands, removed and frozen shortly after death. The glands were allowed to thaw and then kept at room temperature (*ca.* 22°C) for 6 h before lyophilization and extraction. Bottom: RP-HPLC of 200 $\mu$l of acetic acid extract from a normal human pancreas gland removed after 6–8 h ischaemia time. A 280 × 4.6 mm I.D. Dynospheres PD-102-RE column was eluted essentially as in Fig. 1.

TABLE I

CONTENTS OF PROTEIN AND INSULIN (IN PARENTHESES) IN THE CRUDE ACETIC ACID EXTRACTS OF THREE FRESH NORMAL AND FIVE DIABETIC HUMAN PANCREATA, THREE FRESH NORMALS WHICH WERE THAWED AND KEPT AT ROOM TEMPERATURE FOR 6 HOURS BEFORE EXTRACTION AND OF THREE NORMALS WITH A SIMILAR ISCHAEMIA TIME AS THE FIVE DIABETIC GLANDS

The values for the fresh normals and the diabetics are reprinted from ref. 2. The protein content is calculated as mg per gram of lyophilized pancreas and the insulin content as μg per gram of lyophilized pancreas.

| Fresh normal | Fresh normal, 6 h at room temperature | Normal, 6–8 h ischaemia time | Diabetic, 6–8 h ischaemia time |
|---|---|---|---|
| 258 (607) | 269 (9) | 86 (631) | 80 (37) |
| 161 (463) | 325 (14) | 93 (729) | 59 (38) |
| 81 (322) | 311 (1803) | 94 (533) | 15 (15) |
| | | | 41 (19) |
| | | | 53 (14) |

drastically reduced after the incubation period (288 μg per g pancreas compared with 2200 μg per g pancreas for the non-incubated half).

The polypeptide profiles of the two halves (obtained after RP-HPLC using the Dynospheres column) were very similar with respect to the presence of individual sample components, and the SDS-PAGE analyses of the first, second and third extracts were almost identical (Fig. 5). However, larger amounts of most of the separated polypeptides were found in the extract from the fresh half compared with the "incubated" half, and the strongly reduced insulin content in the extracts, as measured in insulin RIA, was clearly demonstrated (Fig. 5).

In order to fully establish the potential of polymeric RP columns for polypeptide separations, acetic acid extracts of a normal and a diabetic pancreas were separated on the Dynospheres column utilizing acetonitrile as organic modifier in combination with two of the most popular mobile phase additives, TFA and TEAP. The resulting chromatograms were further compared with the separation patterns obtained for identical samples and mobile phases using a "classical" silica-based stationary phase, 5-μm 300 Å Nucleosil $C_4$. In order to increase the separation capacity, the gradient time was increased from 1 to *ca.* 2 h, and the separations of the normal and diabetic extracts with TEAP–acetonitrile are shown in Fig. 6 (Dynospheres) and Fig. 7 (Nucleosil).

Similar chromatograms utilizing acetonitrile gradients in TFA are shown in Fig. 8 (Dynospheres) and Fig. 9 (Nucleosil). Although the use of identical gradients resulted in closely comparable chromatograms for extracts of a normal pancreas (top), distinct differences in selectivity were observed in the separations of the diabetic extracts (middle). Several sample components eluted from the Dynospheres column with retention times between 5 and 20 min (Fig. 8, middle) seem to be eluted very early from the $C_4$ column, unresolved from the frontal solvent peak (Fig. 9, middle).

The use of acetic acid gradients in water as mobile phases excludes UV recording of the column eluate at 215–220 nm (as utilized in Figs. 6–9). Extracts of a normal human pancreas were therefore separated using TFA–acetonitrile as mobile

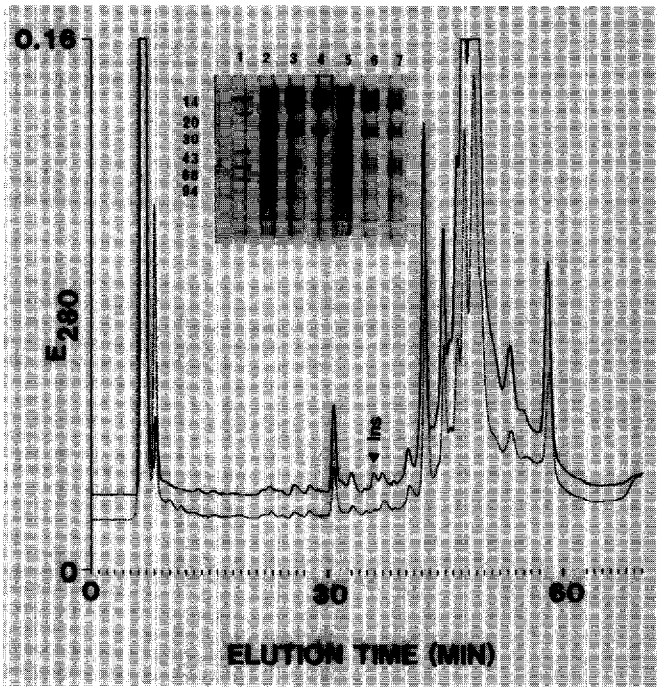


Fig. 5. RP-HPLC of two halves of a normal human pancreas removed and frozen shortly after death. One half was extracted immediately after lyophilization (solid curve) and the other half was kept at room temperature for 6 h before lyophilization/extraction (dotted curve). A 250 × 4.6 mm I.D. Dynospheres PD-102-RE column was eluted essentially as described in Fig. 1. The elution position of authentic human insulin (Ins) (obtained after chromatography of the extract spiked with insulin) is marked. SDS-PAGE of the first, second and third acetic acid extracts of the instantly extracted half (lanes 2, 3 and 4) and of the "incubated" half (lanes 5,6 and 7) is shown as an inset. Lane 1: molecular weight markers; MW in kilodaltons on the left.

phase followed by UV detection at 280 nm for the Dynospheres (Fig. 8, bottom) and the Nucleosil $C_4$ columns (Fig. 9, bottom). When these chromatograms are compared with those obtained after recording at 215 nm (Figs. 8 and 9, top), the chromatograms are strikingly alike. In spite of differences in light intensities for individual sample components, it appears valid to compare results obtained at different UV wavelengths.

## DISCUSSION

We have recently described the use of acetic acid gradients for the analysis of acetic acid extracts of the human pancreas [2]. As the UV profiles of extracts of normal pancreata were very different from those of diabetic samples, and considering the fact that the objective for diabetes is the endocrine part (constituting *ca.* 1% of the weight of the total pancreas), the reason for this difference must originate from elsewhere.

In contrast to the parts of normal human glands (removed and frozen shortly

after death), the diabetic pancreata were obtained as whole organs, removed 6–8 h (or longer) after hospital death. Proteolytic cleavage could be expected to take place under these circumstances, as has been described for insulin, glucagon and pancreatic polypeptide in surgically removed, non-diabetic human pancreata [16–18].

When the RP-HPLC analyses of extracts of individual diabetic glands (Fig. 2) were compared with similar analyses of a normal sample (Fig. 1, bottom), it is evident that the proteolytic cleavage is not restricted to the above-mentioned pancreatic hormones (constituting less than 0.5% of the pancreatic polypeptide mass). Virtually all sample components eluted in the last half of the chromatogram (peak I material, MW >6000 dalton, normally constituting 95–98% of the extractable polypeptides [2]) were degraded. Concurrently with this disappearance, the front peak in the chromatograms increased dramatically, and this elution position would be expected for smaller peptides and amino acids.

The proteolytic degradation was also reflected in the HPSEC profiles for normal and diabetic extracts obtained on both SEC columns (Fig. 3) and in the figures

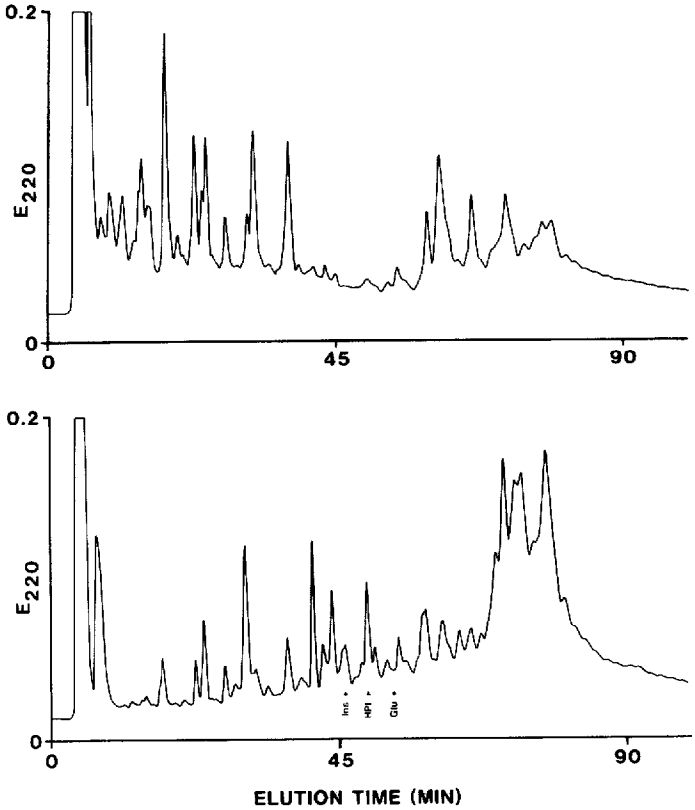


Fig. 6. RP-HPLC of 50 μl of acetic acid extract of a normal (bottom) and a diabetic, human pancreas (top) using a 250 × 4.6 mm I.D. Dynospheres PD-102-RE column eluted with an acetonitrile gradient in 25 m*M* TEAP (pH 3.0) (from 12% to 48% acetonitrile linearly for 100 min). Flow-rate, 1.0 ml/min. The extracts were similar to those analysed in Fig. 1. The elution positions of authentic human insulin (Ins), proinsulin (HPI) and glucagon (Glu) are marked.

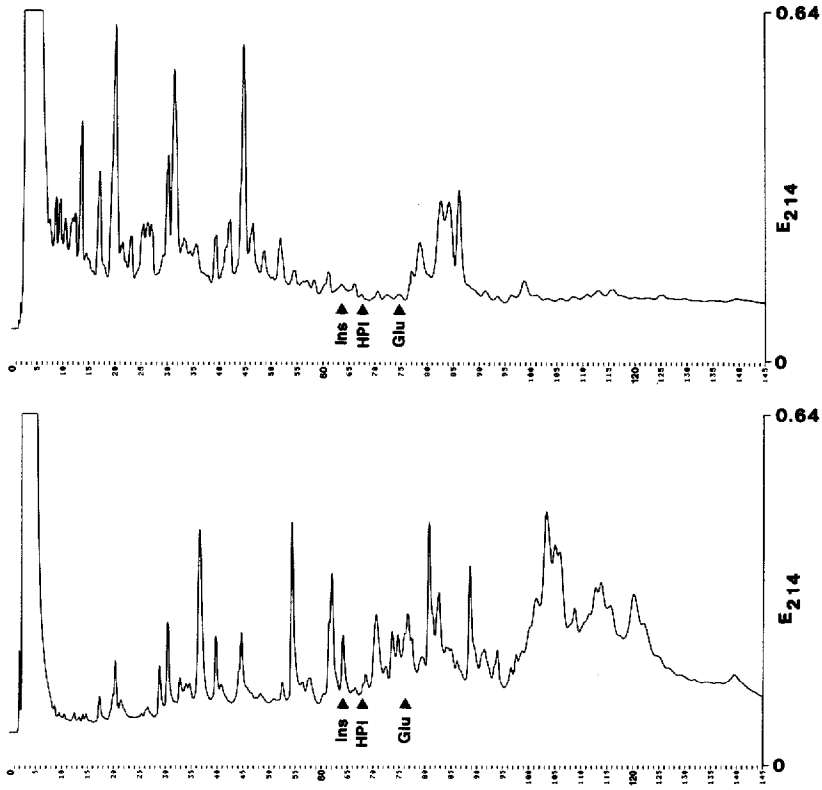


Fig. 7. RP-HPLC of 50 $\mu$l of acetic acid extract of a normal (bottom) and a diabetic human pancreas (top) using a 250 × 4.0 mm I.D. 300 Å Nucleosil $C_4$ (5 $\mu$m) column eluted with an acetonitrile gradient in 25 m*M* TEAP (pH 3.0) (from 12% to 45% acetonitrile linearly for 132 min followed by 45% to 60% acetonitrile linearly for 13 min). Flow-rate, 0.5 ml/min. The extracts were similar to those analysed in Fig. 1. The elution positions of authentic human insulin (Ins), proinsulin (HPI) and glucagon (Glu) are marked. Time scale in min.

for the protein contents in extracts of the five diabetic and three normal pancreatic glands (Table I), although it should be borne in mind, that large individual variations in such biological materials are likely to occur.

From the chromatograms obtained after extracting parts of individual normal pancreata which had been thawed and kept in the laboratory for 6 h at *ca.* 22°C before lyophilization and extraction (Fig. 4, top and middle), it is clear that no enzymatic cleavage comparable to that occurring in the diabetic pancreas was induced. The UV profiles were comparable to those for normal samples, and the protein contents in the raw extracts did not indicate any reduction (Table I). The differences in recoverable polypeptide mass after RP-HPLC and the difference in the size of the front peaks may be ascribed to individual variations in the sample material.

When three whole normal pancreatic glands, obtained after a similar ischaemia time to the diabetic sample, were analysed, the extractable polypeptides were found to be significantly degraded (Fig. 4, bottom). This chromatogram was very similar to those of diabetic glands (Fig. 2), *i.e.*, loss of peak I material and an abundant front peak,

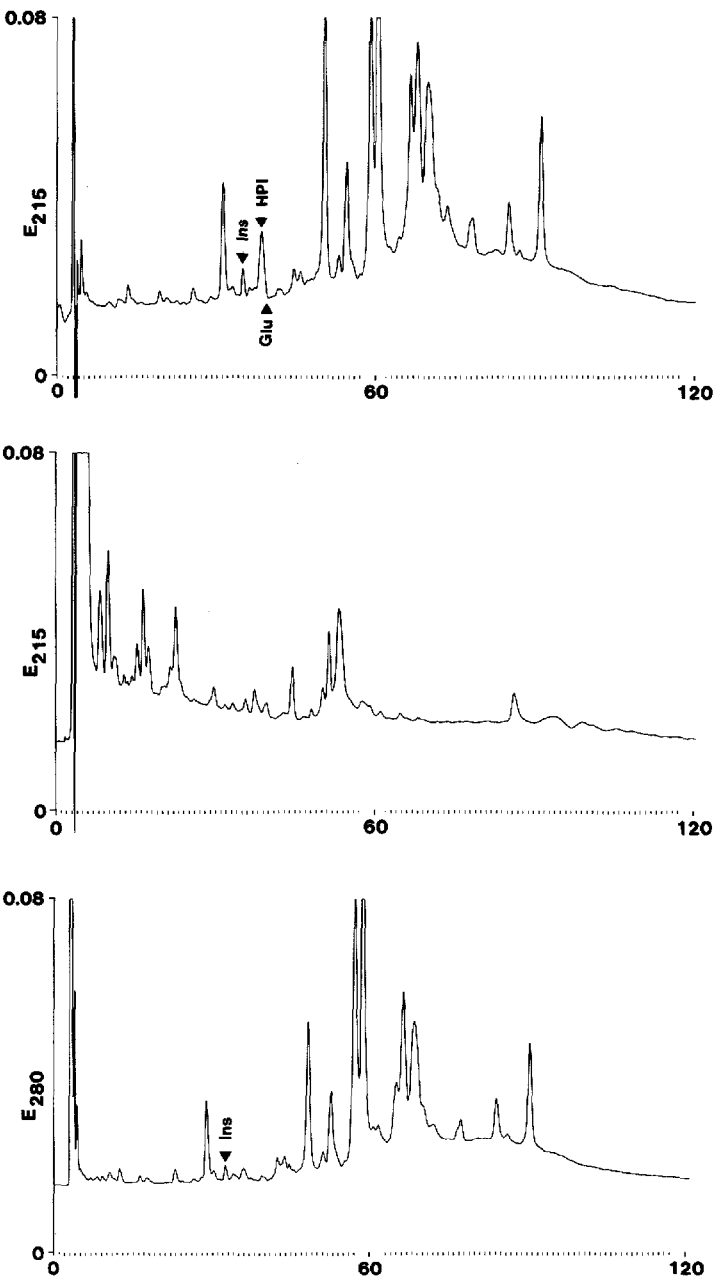


Fig. 8. RP-HPLC of 5 $\mu$l of acetic acid extract of a normal (top) and a diabetic human pancreas (middle) using a 250 × 4.6 mm I.D. Dynospheres PD-102-RE column eluted at 1.0 ml/min with an acetonitrile gradient in TFA (from 20% acetonitrile–0.075% TFA to 60% acetonitrile–0.070% TFA linearly for 120 min). Bottom: RP-HPLC of 50 $\mu$l of acetic acid extract of a normal human pancreas using a 250 × 4.6 mm I.D. Dynospheres PD-102-RE column eluted essentially as described for the top and middle panels. UV detection at 215 nm (top and middle) and 280 nm (bottom). The normal and diabetic samples were identical with those analysed in Figs. 1 and 7. The elution positions of authentic human insulin (Ins), proinsulin (HPI) and glucagon (Glu) are marked.

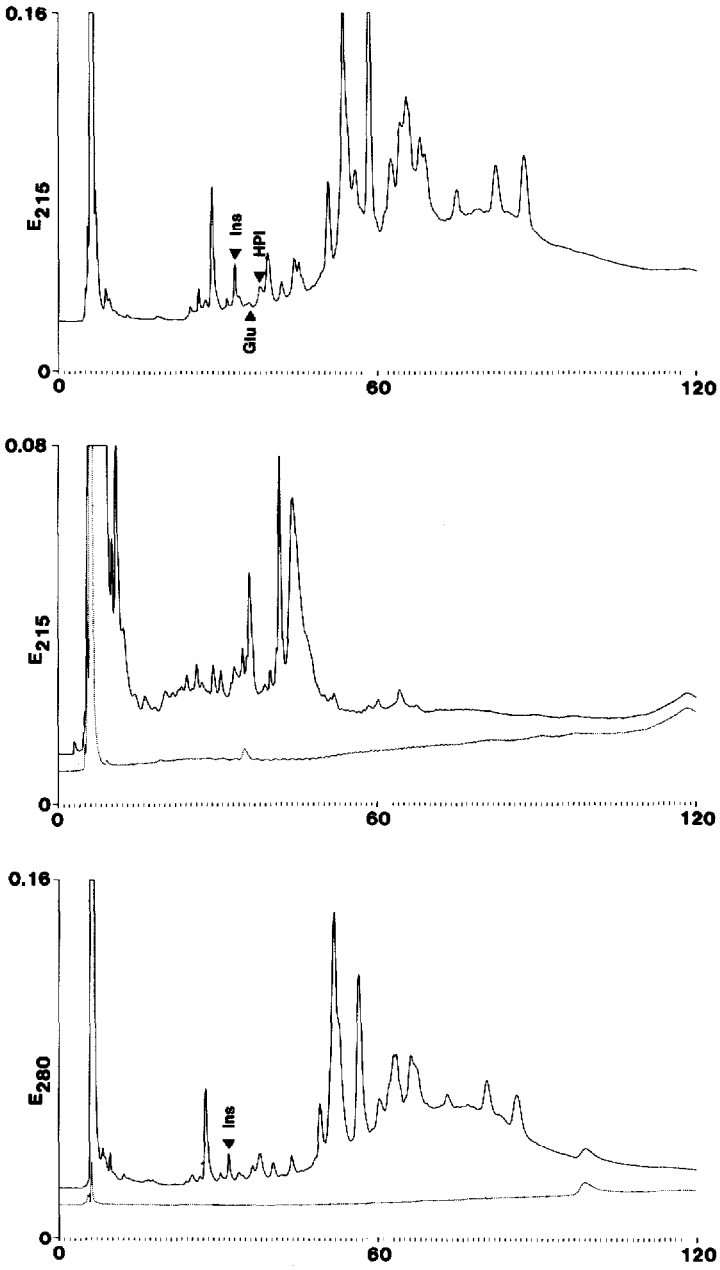


Fig. 9. RP-HPLC of 5 $\mu$l of acetic acid extract of a normal (top) and a diabetic human pancreas (middle) using a 250 × 4.0 mm I.D. 300 Å Nucleosil $C_4$ (5 $\mu$m) column eluted at 0.5 ml/min with essentially the same acetonitrile–TFA gradient as in Fig. 8. Bottom: RP-HPLC of 50 $\mu$l of acetic acid extract of a normal human pancreas using a 250 × 4.0 mm I.D. 300 Å Nucleosil $C_4$ (5 $\mu$m) column eluted essentially as described for the top and middle panels. The lower, dotted curves in the middle and bottom panels represent a blank injection (5 $\mu$l of 3 *M* acetic acid). UV detection at 215 nm (top and middle) and 280 nm (bottom). The normal and diabetic samples were identical with those analysed in Figs. 1, 7 and 8. The elution positions of authentic human insulin (Ins), proinsulin (HPI) and glucagon (Glu) are marked.

and the protein contents in the extracts were of the same order as found in the diabetic extracts (Table I).

The insulin contents in these glands were found to be similar to those of fresh, normal pancreata, whereas the values for fresh normal glands, thawed and kept at room temperature for 6 h, were extremely scattered (Table I). However, a study of 29 pancreatic glands obtained from human non-diabetics with ischaemia times from less than 1 to 24 h failed to show any correlation between the amount of extractable insulin and the corresponding ischaemia time [17]. Further, the insulin content in 32 non-diabetic human pancreata (with ischaemia times $<6$ h) varied from 4 to 192 $\mu$g per g wet pancreas [19], and a marked decrease in the amount of insulin extractable from fresh, immediately frozen pancreata has been reported to occur rapidly on thawing [18].

In spite of these large and expected individual differences among the various pancreata, it can be concluded that the ischaemia time is the major reason for the different UV profiles of extractable polypeptides previously observed between the normal and the diabetic pancreas. If a diabetic pancreas was removed and frozen instantly as for those from healthy persons, the sample constituents in the acetic acid extract would probably be very similar to that of a normal sample, with the exception of the insulin content.

That this difference in insulin content is actually detectable after RP-HPLC analyses using a polymeric column eluted with an acetic acid gradient in water is shown in Fig. 5. The curves represent the extracts of two equal halves of the same (part of a) normal human pancreas, the only difference being that one half was extracted immediately after thawing and the other was kept at 22°C for 6 h before extraction. The insulin content in the extract of the latter half was *ca.* 13% of that in the former, whereas the protein contents in the extracts from the two halves were similar. This difference in insulin content is clearly indicated in the chromatograms (Fig. 5) but, although the protein contents in the two raw extracts were found to be similar, less polypeptide mass is recovered after HPLC in the half left at 22°C. When analysed carefully, the two chromatograms revealed the presence of (various amounts of) identical sample components with retention times between 12 and 60 min, but the front peak was slightly broader, and the three peaks eluted immediately after the front peak were significantly more prominent in the extract from the "incubated" half. These minor differences could indicate an incipient proteolytic degradation after 6 h at room temperature.

Although the two different polymeric columns are based on different monomers (styrene *versus* divinylbenzene) and copolymerized additives (resulting in different loads of aromatics *versus* alkyl groups in the bonded phases), the chromatograms obtained after elution with acetic acid in water of these complex extracts were closely similar. When separations based on this mobile phase were compared with results obtained with an extended acetonitrile gradient in 25 m*M* TEAP, the Dynospheres column (Fig. 6) is seen to change in selectivity: for extracts from a normal pancreas, the resolution of the components in the peak I material (in the last half of the chromatogram) was poor, whereas the part of the chromatogram dedicated to separation of the polypeptides in the peak II material (MW $\leqslant$6000 dalton) became enlarged, and these components were very well resolved. In parallel with this, the extract of a diabetic pancreas showed a much higher heterogeneity than after the acetic acid gradient (compare with Fig. 1).

This change in selectivity was further increased if the polymeric column was replaced with a silica $C_4$ column eluted with the same mobile phase (Fig. 7): the resolution in the final part of the chromatogram of the extract from a normal pancreas was further reduced, whereas the peak shape and the separation capacity obtained for the peak II sample components (eluted in the first half of the chromatogram) were excellent (Fig. 7, bottom). Probably owing to the low hydrophobicity of the $C_4$ ligand, the separation of polypeptides extracted from the diabetic pancreas was less satisfactory in the last half of the chromatogram (Fig. 7, top).

Changing the mobile phase to acetonitrile in TFA resulted in selectivity changes in the opposite direction for the Dynospheres column (Fig. 8): compared with the acetic acid elution shown in Fig. 6, the initial halves of the chromatograms were alike in outline, whereas the peak I material in the last half was very well resolved (top). However, much of the material present in the extract of the diabetic pancreas was now not resolved from the front peak with this mobile phase (bottom), and this lack of resolution of polypeptides with low hydrophobicity was further emphasized when the Dynospheres column was replaced with the Nucleosil $C_4$ column (Fig. 9, top and middle).

When complex polypeptide mixtures are analysed using different stationary and mobile phases, the criteria for success may differ from one way of presenting the problems to another. Judged by parameters such as the ability to resolve the maximum number of components in a single analysis, whether they are characterized by high or low molecular weight and/or hydrophobicity, the use of polymeric RP columns eluted with acetic acid in water appears to be an exciting alternative to classical RP-HPLC polypeptide analyses. This work has further shown that polypeptide selectivity for a polymeric RP column can be manipulated according to documented experiences with mobile phase additives acquired for silica-based alkyl-RP columns.

Studies of the effect of various polymeric stationary phases on the resulting polypeptide selectivity using acetic acid–water and TFA–acetonitrile as mobile phases and the effect of the stationary and mobile phase on polypeptide recovery will be published separately [20].

ACKNOWLEDGEMENTS

We thank Helle Bojesen-Koefoed, Linda Larsø and Ingelise Fabrin for excellent technical assistance.

REFERENCES

1 B. S. Welinder and H. H. Sørensen, *J. Chromatogr.*, 537 (1991) 181.
2 B. S. Welinder and S. Linde, *J. Chromatogr.*, 537 (1991) 201.
3 C. T. Mant and R. S. Hodges, *J. Liq. Chromatogr.*, 12 (1989) 139.
4 K. K. Unger and K. D. Lork, *Eur. Chromatogr. News*, 2 (1988) 14.
5 B. S. Welinder, S. Linde and B. Hansen, *J. Chromatogr.*, 348 (1985) 347.
6 O. D. Madsen, L.-I. Larsson, J. H. Rehfeldt, T. W. Schwartz, Å. Lernmark, O. A. Labrecque and D. F. Steiner, *J. Cell. Biol.*, 103 (1986) 2025.
7 L. Orci, F. Malaisse-Lagae, D. Baetens and A. Perrelet, *Lancet*, ii (1978) 1201.
8 D. Baetens, F. Malaisse-Lagae, A. Perrelet and L. Orci, *Science*, 206 (1979) 1323.
9 F. Malaisse-Lagae, Y. Stefan, L. Cox, L. Perrelet and L. Orci, *Diabetologia*, 17 (1979) 361.
10 J. Rahier, J. Wallen, W. Gepts and J. Hoot, *Cell Tissue Res.*, 200 (1979) 359.

11 L. Orci and L. Perrelet, in R. H. Unger and L. Orci (Editors), *Glucagon, Physiology, Pathophysiology and Morphology of the Pancreatic A-Cell*, Elsevier, New York, 1981, p. 3.
12 E. R. Trimble, P. A. Halban, C. B. Wollheim and A. E. Renold, *J. Clin. Invest.*, 69 (1982) 405.
13 Y. Tasaka, S. Inoue, K. Marumo and Y. Hirata, *Acta Endocrinol.*, 105 (1984) 223.
14 R. L. Gingerich, P. E. Lacy, R. E. Chance and M. G. Johnson, *Diabetes*. 27 (1978) 96.
15 D. J. Gersell, R. L. Gingerich and M. H. Greider, *Diabetes*, 28 (1979) 11.
16 Y. Tasaka, S. Inoue, Y. Harata, F. Hanyu and M. Endo, *Endocrinol. Jpn.*, 28 (1981) 261.
17 J. R. Kimmel and H. G. Pollock, *Diabetes*, 16 (1967) 687.
18 G. A. Wrenshall, C. H. Best and W. S. Hartroft, *Can. J. Biochem. Physiol.*, 35 (1957) 527.
19 G. M. Rastogi, M. K. Sinha and R. J. Dash, *Diabetes*, 22 (1973) 804.
20 B. S. Welinder, *J. Chromatogr.*, 542 (1991) 83.