*Journal of Chromatography*, 369 (1986) 133–141
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 928

# SEPARATION OF GUINEA-PIG PANCREATIC JUICE PROTEINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

P. J. PADFIELD*
*Department of Physiological Sciences, Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT (U.K.)*
M. GRIFFIN
*Department of Biochemistry and Molecular Biology, University of Manchester, Manchester (U.K.)*
and
R. M. CASE
*Department of Physiological Sciences, University of Manchester, Manchester (U.K.)*
(Received July 9th, 1986)

## SUMMARY

Using a Protesil 300 octyl reversed-phase column with a multistage water–acetonitrile solvent gradient system, it was possible to obtain a well-resolved separation of the nine major proteins present in guinea-pig pancreatic juice. The protein present in each peak of the pancreatic juice chromatogram could only be identified by molecular weight analysis as the acetonitrile denaturated the enzymes and altered their isoelectric points. However, using sodium dodecyl sulphate gel electrophoresis the protein fractions obtained by high-performance liquid chromatography were characterised. Preliminary work has indicated that this system may be capable of separating other complex biological protein mixtures, *i.e.*, saliva.

## INTRODUCTION

Hitherto, separation and quantitation of the proteins present in pancreatic juice has been achieved using electrophoretic techniques. Scheele[1] used two-dimensional gel electrophoresis to separate $^{14}$C-labelled proteins secreted by isolated guinea-pig pancreatic lobules. Isoelectric focussing was used in the first dimension and gradient polyacrylamide gel electrophoresis in the second. After electrophoresis and staining, each protein spot was excised from the gel and its radioactivity counted, allowing the mass of protein present in each band to be determined. Dagorn[2] simplified the system by subjecting the pancreatic juice only to isoelectric focussing and by relating the degree of Coomassie blue staining of each band to the mass of protein present. The staining pattern of the gels was measured using a scanning UV spectrophotometer, the quantity of protein present in each band being directly related to its absorbance.

Though the techniques developed by Scheele and by Dargorn do give a sep-

0021-9673/86/$03.50 © 1986 Elsevier Science Publishers B.V.

aration of the major proteins present in pancreatic juice, they have one major drawback. The preparation, running and staining of electrophoretic gels requires skill and is both tedious and time consuming. These techniques are therefore not ideally suited for the analysis of large numbers of samples.

In this paper we describe a separation technique for guinea-pig pancreatic juice proteins based on reversed-phase high-performance liquid chromatography (HPLC). Using one of the relatively new, wide-pore (300 Å), reversed-phase columns specifically designed for protein separation it is possible to separate pancreatic secretory proteins. Coupled with UV spectrophotometric analysis of the eluted proteins, this allows simple, accurate and reproducible quantitation of the proteins present in pancreatic juice.

A report of the initial work was given at the 1984 meeting of the European Pancreatic Club[3].

## EXPERIMENTAL

### *Analytical HPLC*

*Equipment.* All chromatography was performed on a Varian (Palo Alto, CA, U.S.A.) 5020 liquid chromatography system. The eluted proteins were detected by a Varian UV50, UV–visible, variable-wavelength detector and the resulting chromatograms were printed out either by a Varian 9176 chart recorder or a Varian Vista 402 integrator system.

*Column.* A Protesil 300 octyl, 250 × 4.8 mm reversed-phase column was used (Whatman, NJ, U.S.A.).

*Solvent system.* Water and acetonitrile, each containing 0.1% (v/v) trifluoroacetic acid (TFA), were run as a gradient system, the details of which, with their flow-rates, are given in the legends to the figures.

*Preparation of samples.* The samples of pancreatic juice were untreated. However, their protein concentrations were determined and adjusted to 1.0 to 1.5 mg/ml by addition of deionized water. Samples were loaded onto the column via a fixed-volume (10 μl) loop injector.

### *Preparative HPLC*

*Equipment.* The chromatography system, detector and integrator were as stated above. Eluted fractions from the column were collected in 0.2-min timed volumes using an LKB Ultrorac 7000 fraction collector. The void volume between the flow cell of the detector and the fraction collector was approximately 50 μl.

*Column.* A Protesil 300 octyl, 250 × 9 mm reversed-phase column was used (Whatman).

*Solvent system.* Water and acetonitrile, each containing 0.1% (v/v) TFA, were run in a gradient consisting of five steps. Flow was initiated at 0% acetonitrile, 100% water. The concentration of acetonitrile was increased at a rate of 12%/min for 3 min. After 3 min, the rate of acetonitrile addition was changed to 0.5%/min for 8 min, 0%/min for 4 min, 0.5%/min for 18 min and finally to 2%/min for 12 min. This gradient is identical to the one found to produce the best separation on the analytical Protesil column (see Results). However, the flow-rate was increased from 1.5 to 6.2 ml/min to compensate for the larger volume of the column.

*Preparation of samples.* Pancreatic juice samples for the preparative runs were obtained by freeze drying bulk volumes of guinea-pig pancreatic juice. The dried protein was redissolved in deionized water to give a final protein concentration of 50 mg/ml and 500 $\mu$l of this solution were used for each run.

*Storage of collected fractions for electrophoresis.* After collection the fractions were freeze dried and stored at $-80$°C.

*Collection of pancreatic juice.* Male guinea-pigs weighing 400 to 500 g which had been denied food overnight were anaesthetized with sodium pentobarbitone (30 mg/kg, i.p.). Through an abdominal incision the main pancreatic duct was then cannulated and the pylorus ligated to prevent gastric contents entering the duodenum. Pancreatic secretion was stimulated by a constant infusion of synthetic porcine secretin (2.0 $\mu$g/kg · h) via a catheter in the jugular vein. Pancreatic juice was collected for 20 consecutive 5-min periods and stored at $-4$°C.

*Sodium dodecyl sulphate (SDS) gel electrophoresis*

SDS gel electrophoresis was carried out according to the method of Laemmli[4]. The running gel contained 0.1% (v/v) SDS, 0.375 *M* Tris–HCl, pH 8.8, and either 10 or 15% (v/v) acrylamide. Polymerization was initiated by the addition of 0.025% (v/v) tetramethylethylenediamine (TEMED) and was complete within 1 h. The gels were formed in a Perspex mould (dimensions 90 × 120 × 2 mm).

*Preparation of protein samples.* The protein samples (1.0 mg/ml concentration) were boiled for 15 min with an equal volume of buffer containing 2.5 m*M* Tris–HCl, pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (v/v) bromophenol blue. Samples were delivered to the gel slots in 40-$\mu$l quantities. The loaded gel was run with a current of approximately 5 mA, the current being adjusted occasionally to allow the gels to run within 3 h.

*Staining procedure.* Gels were stained by immersing them for 1 h in an aqueous solution of 5% (v/v) sulphosalicyclic acid, 5% (v/v) trichloroacetic acid (TCA), 30% (v/v) isopropanol and 0.12% (v/v) Coomassie blue. The stained gels were then destained by placing them in an aqueous solution containing 30% (v/v) isopropanol and 10% (v/v) acetic acid. After destaining the gels were stored under water at 22°C.

*Materials*

All the chemicals used for the electrophoresis were obtained from Sigma, Poole, U.K. Synthetic secretin used was supplied by Cambridge Research Biochemicals, Marston, U.K. The solvents used for the HPLC were purchased from Rathburn, Walkerburn, U.K.

## RESULTS

Initially a very crude separation of guinea-pig pancreatic juice proteins was obtained using a relatively steep elution gradient (4%/min) (Fig. 1). By adjusting the rate of addition of the acetonitrile and changing the solvent flow-rate, the preliminary separation was improved to that shown in Fig. 2. The gradient alterations consisted of slowing to different degrees various segments of the original gradient. To combat the peak-broadening effect of slowing the addition of acetonitrile, the eluent flow-rate was increased from 1.0 to 1.5 ml/min.

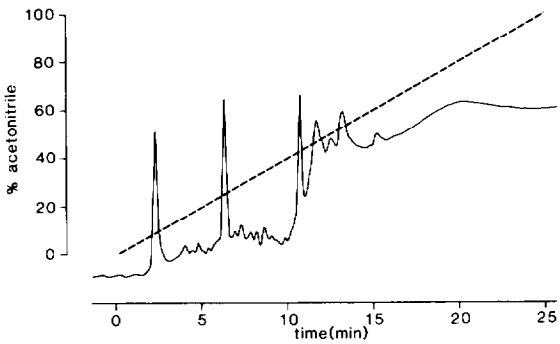


Fig. 1. The separation obtained on placing 10 $\mu$l of secretin-stimulated guinea-pig pancreatic juice on the Protesil 300 octyl (250 × 4.8 mm) reversed-phase column. Conditions: flow-rate 1.0 ml/min; solvent, water–acetonitrile; gradient, acetonitrile increasing linearly at the rate of 4%/min (denoted by the broken line); detection wavelength, 206 nm.

After the system had been optimized for the separation of guinea-pig pancreatic juice proteins, each of the eluted peaks was identified. Characterization of the eluted proteins by their biological activity or isoelectric point proved impossible because the strong denaturing effect of acetonitrile destroyed the enzymatic activity and altered the isoelectric point of the pancreatic enzymes[5]. One parameter which was not affected by the eluting solvent was protein molecular weight. Consequently the protein constituent of each of the eluted peaks was identified on the basis of its molecular weight using SDS gel electrophoresis.

Pure samples of each of the chromatographic peaks were obtained using a preparative form of the analytical column. The chromatogram obtained from the larger column is shown in Fig. 3. To minimize the problem of contamination from adjacent peaks, only mid-peak fractions were collected for analysis. Initially all the peak fractions were run on 10% SDS gels. From these it was possible to observe

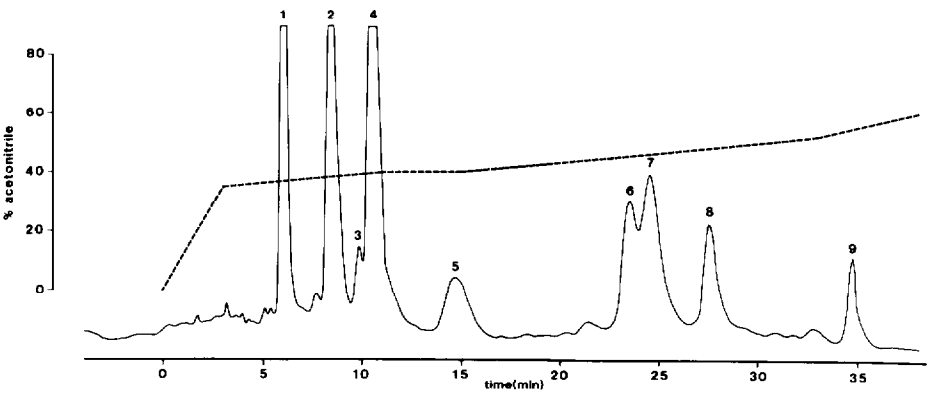


Fig. 2. The optimal separation obtained on placing 10 $\mu$l of secretin-stimulated, guinea-pig pancreatic juice on the Protesil 300 octyl (250 × 4.8 mm) reversed-phase column. Conditions: flow-rate, 1.5 ml/min; solvent, water–acetonitrile; gradient, 5 steps, denoted by the broken line; detection wavelength, 206 nm. The sloping baseline was eliminated by the use of the Vista 402 integrator. For peak identification, see Table I.

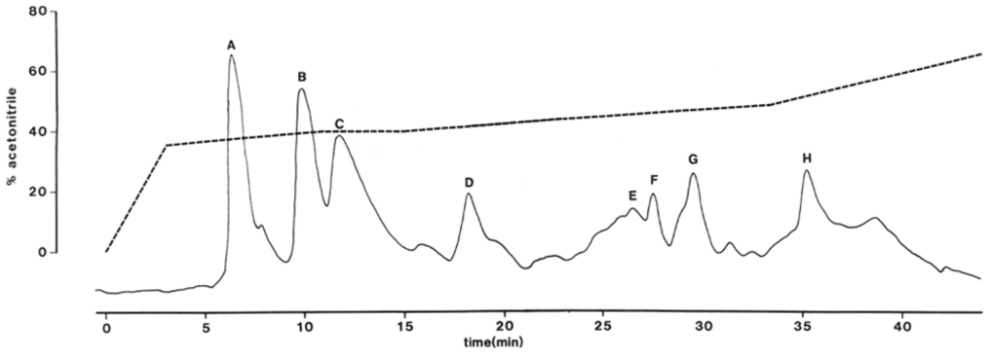


Fig. 3. The separation obtained from 500 $\mu$l of guinea-pig pancreatic juice placed on the Protesil 300 octyl (250 × 9 mm) reversed-phase column. Conditions: flow-rate, 6.2 ml/min; solvent, water–acetonitrile; gradient, 5 steps, denoted by the broken line; detection wavelength, 206 nm. The sloping baseline was eliminated by the use of the Vista 402 integrator. For peak identification, see Table I.

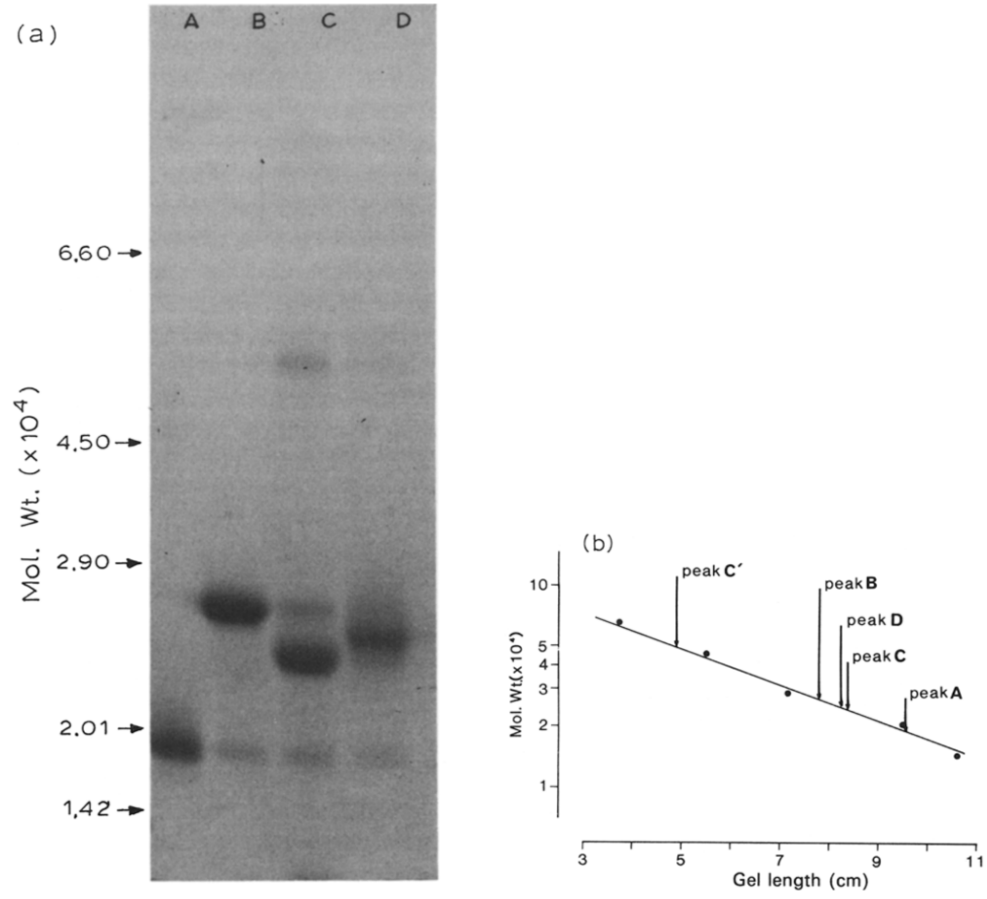


Fig. 4. (a) Sodium dodecyl sulphate electrophoresis on a 15% polyacrylamide gel of the unreduced isolated fractions from peaks A, B, C and D from the preparative HPLC run shown in Fig. 3. Running time 3 h; protein load, 20 $\mu$g per track. The positions of the molecular weight standards are represented on the left hand edge of the gel. (b) Semi-log plot of molecular weight *versus* gel length for the non-reduced molecular weight standards.

which of the samples contained low-molecular-weight proteins (15 000 to 30 000) and which contained the larger proteins (30 000 to 70 000). This was important because the 10% SDS gels did not have the required sensitivity to analyse the lower-molecular-weight proteins. Therefore the peak samples containing the lower-molecular-weight fractions were re-run on 15% SDS gels, and the larger proteins were run again on 10% SDS gels (Figs. 4 and 5).

From the gels it was apparent that, even though mid-peak fractions were collected, contamination from adjacent peaks still occurred. In general the minor bands resulting from this cross-contamination were ignored and the main band used for molecular weight determination. However, there was one exception. In the case of track C (Fig. 4), in addition to the usual contamination, there was also an extra

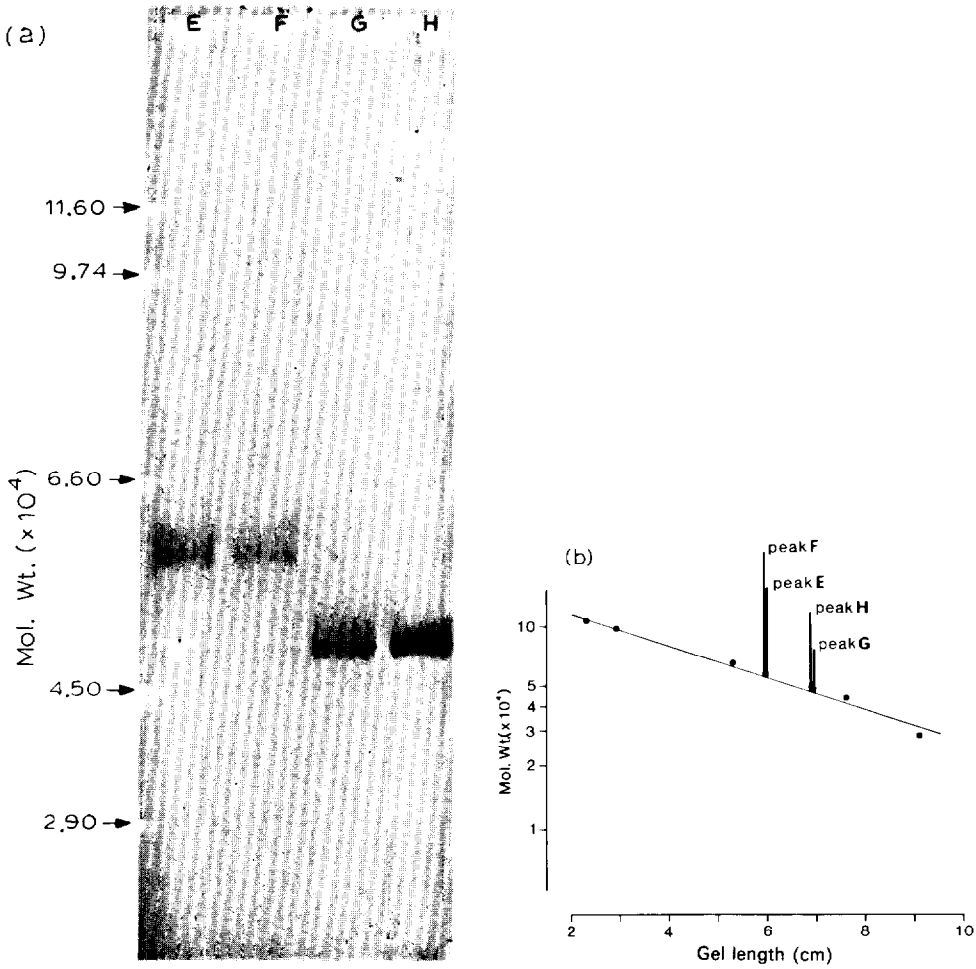


Fig. 5. (a) Sodium dodecyl sulphate electrophoresis on a 10% polyacrylamide gel of the unreduced isolated fractions from peaks E, F, G and H from the preparative HPLC run. Running time 3 h; protein load, 20 μg per track. The positions of the molecular weight standards are represented on the left-hand edge of the gel. (b) Semi-log plot of molecular weight *versus* gel length for the non-reduced molecular weight standards.

TABLE I

MOLECULAR WEIGHTS (OBTAINED BY SDS ELECTROPHORESIS) OF THE PEAK FRACTIONS COLLECTED FROM THE PREPARATIVE HPLC COLUMN

For identification the molecular weight values obtained are compared with the values obtained by Scheele[1] for the guinea-pig pancreatic enzymes.

| *Peak No.* | | *Molecular weight* | | *Identity* |
|---|---|---|---|---|
| *Analytical HPLC* | *Preparative HPLC* | *This study* | *Scheele* | |
| 1 | A | 19 000 | 18 750 | Trypsinogen |
| 2 | B | 28 300 | 28 200 and 28 700 | Proelastase |
| 3 | C′ | 47 200 | 47 600 | Lipase |
| 4 | C | 24 600 | 25 000 | Chymotrypsinogen 1 |
| 5 | D | 25 200 | 25 000 | Chymotrypsinogen 2 |
| 6 | E | 52 000 | 51 000 | Amylase |
| 7 | F | 54 300 | 54 000 | Amylase |
| 8 | G | 45 200 | 45 100 and 45 300 | Procarboxypeptidase A |
| 9 | H | 46 000 | 46 000 | Procarboxypeptidase B |

protein band (C′) with a molecular weight of 47 200. This may represent peak 3 on the analytical chromatogram (Fig. 2) which was not resolved in the preparative run (Fig. 3), probably because it was masked by the much larger B and C peaks. The molecular weight of each of the isolated peak fractions are listed in Table I.

The chromatogram from the preparative column (Fig. 3) was different from that obtained using the analytical column (Fig. 2) due to inter-column variance and the size difference between the columns. Overall the elution profiles were similar but the elution times were altered. Therefore, to match each peak on the preparative chromatogram with its associate on the analytical chromatogram, a sample of each of the collected fractions from the preparative column was loaded, in turn, onto the smaller protesil column. The resulting chromatograms were compared with a standard separation of whole guinea-pig pancreatic juice. The main peak from each preparative fraction could then be directly related to a peak in the analytical chromatogram (Fig. 6). Using this procedure each of the isolated peak fractions was assigned to a peak on the analytical chromatogram (see Table I). Close examination of the trace obtained from fraction C shows it to contain a peak which is comparable to peak 3 on the analytical chromatogram. This would appear to confirm the suggestion that the extra protein band observed in track C of the 15% SDS gel is due to this peak.

The above maneouvres allowed the molecular weights of the protein present in each of the peaks eluted from the analytical column to be obtained. To identify these proteins their molecular weights were compared with those of known guinea-pig pancreatic juice proteins[1]. This comparison is shown in Table I.

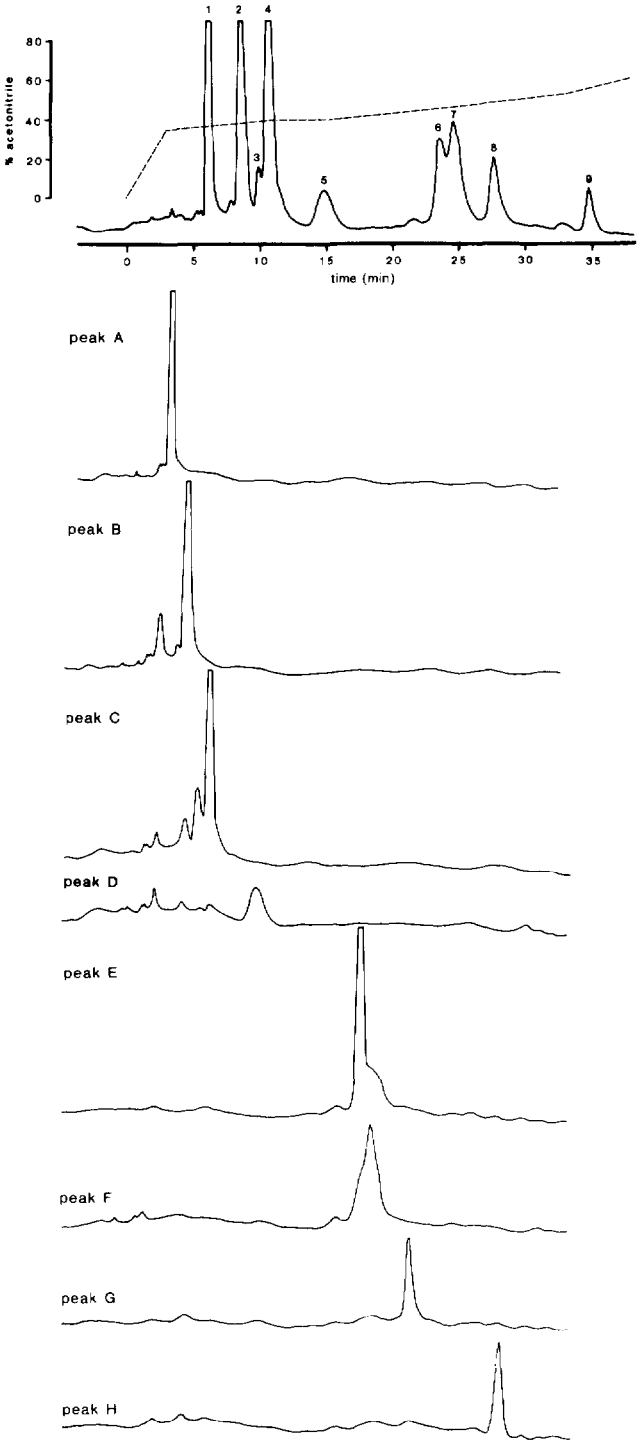


Fig. 6. Comparison of the chromatograms produced from 10-$\mu$l samples of whole guinea-pig pancreatic juice and the mid-peak fractions collected during preparative HPLC. All separations were obtained using the Protesil 300 octyl (250 × 4.8 mm) reversed-phase column. Conditions: flow-rate, 1.5 ml/min; solvent, water–acetonitrile; gradient 5 steps, denoted by the broken line on the top chromatogram; detection wavelength 206 nm.

## DISCUSSION

Initially we determined the feasibility of using a gel permeation system to obtain a separation of pancreatic juice proteins. However, the columns tested did not have the resolution required to separate the individual secretory enzymes. Consequently we experimented with reversed-phase HPLC. Using a wide-pore (300 Å) $C_8$ reversed-phase column with a water–acetonitrile gradient elution system it is possible to obtain a clear separation of the main protein constituents in guinea-pig pancreatic juice. Other solvent pairs were tested (water–methanol, triethylamine acetate–propanol) but these did not produce any clear separations.

Though the reversed-phase mode of HPLC does have a high power of resolution, it has one severe drawback: the solvents used to produce the separations often have deleterious effects on the proteins they elute, making characterization of the separated proteins difficult. Unfortunately this was true of acetonitrile: its strong hydrophobic nature and low pH altered the isoelectric points and destroyed biological activity of the pancreatic juice enzymes. However, the solvent does not appear to change a protein's molecular weight. Therefore, using SDS electrophoresis, it was possible to determine the molecular weight of each of the major protein peaks present in the analytical chromatogram. By comparing these values with the known[1] molecular weights of the guinea-pig pancreatic juice enzymes, it was possible to identify each of the peaks observed in Fig. 2.

As the molecular weights obtained for the eluted protein peaks were very close to values determined by Scheele[1], identification of each of the peaks could be made with some certainty. A second method of characterizing the identity of these peaks is needed to confirm this study. At the present time we believe this study is of value in its own right since it can be applied to other protein systems equally well. It has the big advantage that no biological property of the proteins is being assayed, since fractionation and characterization depends solely on the physical properties of individual proteins.

In conclusion, the reversed-phase HPLC method we describe allows rapid, accurate separation of the major constituents of guinea-pig pancreatic juice. Preliminary observations suggest that this method is applicable to pancreatic juice from human, cat, rat and hamster and to saliva from rabbit mandibular gland, although the system must be optimized for each of these examples.

## ACKNOWLEDGEMENT

P.J.P. was supported by the Peter Kershaw Trust.

## REFERENCES

1 G. A. Scheele, *J. Biol. Chem.*, 250 (1975) 5375.
2 J. C. Dagorn, *Biochim. Biophys. Acta*, 280 (1978) 680.
3 P. J. Padfield and R. M. Case, *Digestion*, 30 (1984) 76.
4 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
5 P. J. Padfield, unpublished results.