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Separation of rat pancreatic secretory proteins by cation-exchange fast protein liquid chromatography☆

Martin J. Fuchs and Volker Keim*

Medizinische Klinik, Klinikum Mannheim, Universität Heidelberg, Theodor-Kutzer Ufer, W-6800 Mannheim (Germany)

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ABSTRACT

Rat pancreatic secretory proteins were separated by an automated liquid chromatography system utilizing a Mono S cation-exchange column. Optimal resolution was obtained with a multistep salt and pH gradient (0.01–2 *M* LiCl, pH 5.3–63.). A total of fourteen well-separated peaks, as well as several minor peaks, were detected by UV absorption. The main pancreatic enzymes were resolved (two amylases, two chymotrypsinogens, two trypsinogens, proelastase, lipase, prophospholipase A_2, procarboxypeptidase A, procarboxypeptidase B, and ribonuclease). In addition, proteins without enzymic activity, such as lithostathine and pancreatitis-associated protein, were identified. Activation of proenzymes did not occur during the separation. At a flow-rate of 0.5 ml/min, *ca.* 250 μg to 5 mg of protein could be applied with equal resolution. The reproducibility of retention volumes and peak areas was high (less than 1% or 5% variation, respectively). When radiolabeled proteins were separated, a comparable pattern of peaks was obtained. The technique described is, therefore, not only useful for analytical and preparative separation of pancreatic proteins but can additionally serve for quantitative determination of the pancreatic isoenzyme pattern.

INTRODUCTION

The development of the two-dimensional gel electrophoresis by O'Farrell [1] and Scheele [2] enabled the analysis of complex protein mixtures, such as the secretory proteins of the pancreas [2–6]. However, quantitative determination of proteins by this technique is hampered by several problems, including loss of material during transfer from the first to the second dimension gel, unequivocal staining of proteins, or complicated and expensive computer-assisted evaluation techniques. When radiolabeled proteins have to be analysed, in most cases the individual spots must be cut out and the gel pieces solubilized. These techniques are not easy to perform, especially when large amounts of sample have to be processed. Simpler methods, such as one-dimensional gel electrophoresis, have been successfully used for several purposes, including determination of pancreatic isoenzyme compositions [7,8], isoenzyme synthesis [9], and measurement of asynchronous intracellular protein transport in the pancreas [10]. These methods, however, only incompletely resolve the pancreatic protein pattern. To make further progress, several high-performance liquid chromatographic (HPLC) techniques have been employed to separate pancreatic secretory proteins in rats, rabbits and humans [11–14]. The analysis is complete in *ca.* 30–45 min, but the separation of proteins remains incomplete.

This paper describes a more efficient chromatographic technique for the resolution of rat pancreatic secretory proteins by automated fast protein liquid chromatography (FPLC). The

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method combines the advantages of HPLC technology (fast run, easy protein determination by UV recording) with those of two-dimensional gel electrophoresis (high resolution). The variable application volume, high loading capacity of the column, high recovery of enzyme activity and absence of proenzyme activation during the separation support the versatility of this method.

EXPERIMENTAL

All reagents were obtained from Sigma (Munich, Germany) unless otherwise indicated. The FPLC system, including a controller LCC500 plus, two pumps P500, UV-photometer UV1, fraction collector FRAC100 and motor valve MV7, were purchased from Pharmacia-LKB (Freiburg, Germany).

Buffers and sample preparation

For the separation of proteins two different buffer systems were used: buffer A (application buffer), 0.05 *M* morpholinoethansulphonic acid (MES) and 0.01 *M* LiCl (pH 5.0–5.5); buffer B, 0.05 *M* MES and 2 *M* LiCl (pH 6.0–6.5). The pH was adjusted with 2 *M* LiOH, and the solution was filtered through a 0.22-μm filter (Braun Melsungen, Germany).

Pure pancreatic juice was collected from conscious rats as described previously [7]. In other experiments rats were fed with three different diets for 10 days (carbohydrate-poor diet: 5% carbohydrates (ch), 12% fat (f), 57% protein (p); fat-rich diet: 26% ch, 38% f, 24% p; protein-rich diet: 33% ch, 5% f, 50% p; for exact composition see ref. 15). With respect to protein, carbohydrate and fat, the protein-rich diet is similar to normal rat chow, but its composition is much better defined.

Under ether anaesthesia, 50 μCi of [^{35}S]methionine (Amersham-Buchler, Braunschweig, Germany, specific activity greater than 800 Ci/mmol) was injected subcutaneously into rats, and zymogen granules were prepared after 60 min [16]. To analyse pancreatic enzymes from pancreatitic rats, acute experimental pancreatitis was induced 48 h prior to the injection of the radiolabeled methionine [17].

Aliquots of pancreatic juice containing *ca.* 500 μg of protein were mixed with the application buffer (usually *ca.* 100–500 μl of sample plus 500–2500 μl of buffer A), the mixture was filtered through the 0.22-μm filter system, and 500–2500 μl were applied to the column.

Fractionation of proteins from pancreatic juice

For analysis, a Mono S HR 5/5 column (Pharmacia) was used. Several different gradients, different salt concentrations, and flow-rates were tested in order to achieve optimal separation of pancreatic proteins. Monitoring was by measurement of the absorption at 280 nm. The eluate was collected on ice and was frozen immediately. At the end of each run the column was washed with 4.5 ml of buffer B. After five to ten separations the column was rinsed with 2 *M* NaOH, 0.5% sodium dodecyl sulphate (SDS) or 70% formic acid.

Identification of proteins in the eluate

The enzymic activities of amylase, chymotrypsin, trypsin, elastase, carboxypeptidase A, carboxypeptidase B, lipase, and ribonuclease were measured as described recently [12]. To determine the enzymic activity of phospholipase A_2, a commercially available assay system was used (Boehringer Mannheim, Germany). To demonstrate the absence of free proteolytic activity, non-activated and trypsin- or enterokinase-activated eluate was assayed for the respective enzymic activities. With these methods, activation of less than 0.1% of the respective proenzymes could be detected. Purified standard rat lithostathine and pancreatitis-associated protein (PAP) were used to determine the elution volumes of these proteins [18]. PAP was also measured by enzyme-linked immunosorbent assay (ELISA) as described recently [18].

Gel electrophoresis

The eluted peaks were collected individually and lyophilized. After reduction with 2% dithiothreitol and carboxymethylation with 2% iodoacetamide (final concentrations, respectively), the samples were loaded on SDS gels (12% acryl-

amide). After an overnight run the bands were stained with the Coomassie-enhanced silver-staining method [19]. In brief, the gels were consecutively rinsed with ethanol, water and 0.1% silver nitrate. The staining was developed in 0.03% formaldehyde, and the reaction was stopped with 1% glycine. The gels were stored in 10% glycerol–0.01% NaN_3. For isoelectric focusing (IEF) or two-dimensional (2D) gel electrophoresis the column eluates were desalted with an NP-5 column (Pharmacia). Ultra-thin-layer IEF was performed with commercially available gels (Precots 3.5-10; Serva, Heidelberg, Germany). The 2D gel electrophoresis was performed as described recently [18].

Recovery and reproducibility

Samples of pancreatic juice containing 320 μg or 1.6 mg of secretory proteins were separated eight or four times, respectively, by FPLC under identical conditions. From the recorded UV absorption spectrum the retention volumes (V_R) of the peak maxima were determined. The areas under the respective peaks were measured by triangulation (height of the peak times width at median height). To account for the recovery the respective proteins, enzyme activities were measured in the sample applied onto the column as well as in the eluate. The recovery of ^{35}S-labeled proteins was determined similarly.

RESULTS

An optimal resolution of rat pancreatic juice secretory proteins was obtained with the multistep pH and salt gradient shown in Table I. Buffer A was used at pH 5.3, and buffer B at pH 6.3. Fourteen major peaks were detected by UV absorption (Fig. 1, for peak 7 see below). Several minor peaks, which did not appear regularly, were noted (unlabeled peaks in Fig. 1). Alteration in the gradient shape, the pH of buffer A (varied from 5.0 to 5.5) or the pH of buffer B (varied from 6.0 to 6.5) did not improve the separation of the proteins. By contrast, when the pH of buffer A was changed to 5.2 or 5.4, the resolution of peaks 3 to 5 and 8 to 10 was incomplete. It

TABLE I

SOLVENT GRADIENT FOR SEPARATION OF RAT PANCREATIC SECRETORY PROTEINS

Elution volume (ml)	Buffer B (%)[a]
0–4	0–2.7
4–6	2.7
6–9	2.7–4.7
9–11	4.7
11–17	4.7–9
17–19	9
19–20	9–21
20–28	21–50
28–28.5	50–100
28.5–32	100
32–37	0

[a] 0.05 *M* MES, 2 *M* LiCl (pH 6.3).

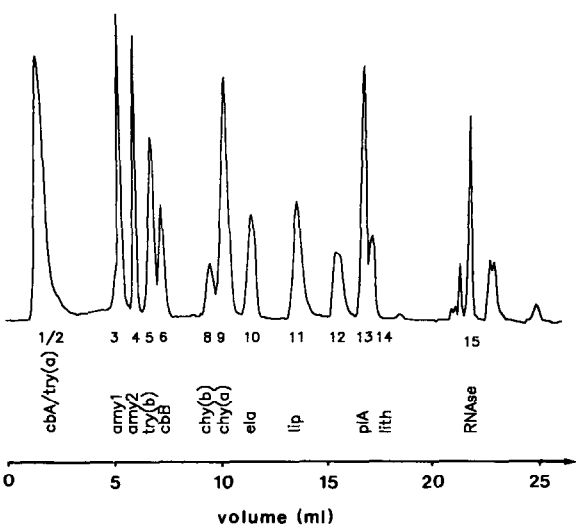

Fig. 1. Separation of rat pancreatic secretory proteins by ion-exchange chromatography. Secretory proteins from rat pancreas were separated by ion-exchange chromatography on a Mono S column using an FPLC system. The separation was achieved by a multistep pH and salt gradient (buffer A, 0.05 *M* MES, 0.01 *M* LiCl, pH 5.3; buffer B, 0.05 *M* MES, 2.0 *M* LiCl, pH 6.3; for details of the gradient see Table I) at a flow-rate of 0.5 ml/min. The separation was monitored by measurement of the UV absorption of the eluate at 280 nm. Abbreviations: cbA = [pro]carboxypeptidase A; try(a) = acidic trypsin[ogen]; amy 1 + 2 = amylase 1 + 2; try(b) = basic trypsin[ogen]; cbB = [pro]carboxypeptidase B; chy(b) = chymotrypsin[ogen] (basic); chy(a) = chymotrypsin[ogen] (acidic); ela = [pro]elastase; lip = lipase; plA = [pro]phospholipase A_2; lith = lithostathine; RNAse = ribonuclease.

is remarkable that these relatively small alterations in the pH of the buffer have such a considerable influence on the separation of proteins. The resolution could not be improved by use of other buffers or salts, such as hydroxyethylpiperacilline-ethansulphonic acid (HEPES), NaCl, or $CaCl_2$.

The separation was optimal at a flow-rate of 0.5 ml/min. At lower rates the number of peaks was unaltered, but at 1.0 ml/min several fused peaks appeared. The sample application volume was varied from 0.5 to 2.5 ml without significant influence on the resolution. For a normal run *ca.* 500 μg of protein were applied. This amount could be increased to 5 mg with no change of the separation pattern. When less than 250 μg of protein were used, the UV absorption spectrum was difficult to evaluate because of baseline variation.

When the enzymic activity in the eluate was measured, two well-separated peaks representing amylase (peaks 3 and 4) could be demonstrated (Fig. 1 and 2). Furthermore, single peaks with lipase (peak 11) and ribonuclease (peak 15) activities were found. However, though the technique enabled the measurement of activation of less than 0.1% of the proenzymes, no free proteolytic activity was detectable in the column eluate. The proenzymes were therefore activated by addition of enterokinase or trypsin. Two trypsins (peaks 1/2 and 5), two chymotrypsins (peaks 8 and 9), elastase (peak 10), carboxypeptidase A (peak 1/2), carboxypeptidase B (peak 6), and phospholipase A_2 (peak 13) were identified (Figs. 1 and 2). Rat lithostathine was detected by elution of a purified standard preparation. The protein was found at V_R = 17.5 ml (peak 14). Minor enzymic activities (less than 10% of the main form) were noted for carboxypeptidase B (V_R = 8.5 ml) and elastase (12.8 ml) without clear association with a protein peak. No enzymic activity could be attributed to the proteins in peak 12.

After induction of experimental pancreatitis, the rat pancreas synthesizes and secretes a protein termed pancreatitis-associated protein or PAP, which is not present in pancreatic juice or zymogen granules form normal rats [18]. To detect this compound, zymogen granule proteins

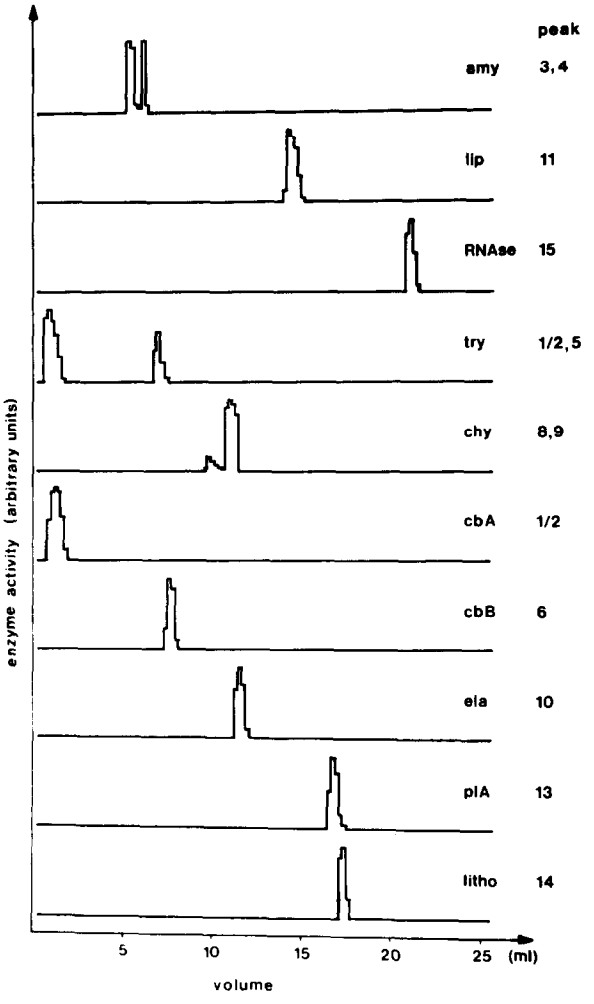

Fig. 2. Enzymic activities in the eluate after separation of pancreatic proteins. Pancreatic proteins were separated by FPLC as above, and the eluate was collected in 0.25-ml fractions. Activities of pancreatic enzymes were measured. For identification of rat lithostathine (lith) the pure protein was run under identical conditions. For abbreviations and peak numbers see Fig. 1.

from rats with experimental pancreatitis were separated. At a retention volume of 8.5 ml an additional protein (peak 7) was found (Fig. 3A) which is absent in normal controls (see Fig. 1). Chromatography of a standard preparation of purified rat PAP revealed a single peak at an identical position (Fig. 3B). When analysed by a specific ELISA, the PAP immunoreactivity was also associated with this protein peak (not shown).

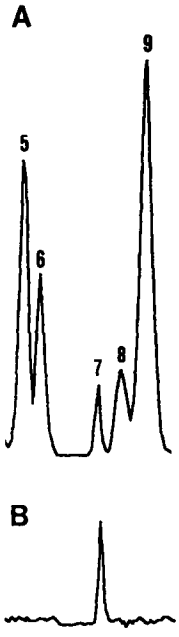

Fig. 3. Identification of the "pancreatitis-associated protein" (PAP) in the eluate. Zymogen granule proteins from rats prepared 48 h after induction of acute pancreatitis were separated by FPLC. Only a part of the UV pattern is shown (A). For comparison, pure PAP (40 μg) was run under identical conditions (B).

To confirm the identification of individual peaks, the column eluates were collected and lyophilized, and the proteins were separated by SDS gel electrophoresis. Nearly all peaks represented a single band after stainig of the SDS gel (not shown). When the apparent molecular masses of the bands were compared with literature values, no important difference was found (Table II).

In order to identify acidic and basic isoforms of the individual (pro)enzymes, the proteins were separated by ultra-thin-layer IEF and by 2D gel electrophoresis. The results of some experiments are shown in Fig. 4. The isoelectric points of the proteins were determined by comparison with commercially available standards (Fig. 4F), and a good agreement was found with literature values (Table II). The acidic proteins, however, do not always elute before the basic isoforms (acidic chymotrypsinogen: peak 9, basic chymotrypsinogen: peak 8). In addition, amylase with an isoelectric point above 8.5 elutes very early (peaks 3 and 4). Therefore, the correlation coefficient between the retention volumes and the isoelectric points of the proteins was only 0.58.

When pancreatic proenzymes were activated by addition of trypsin (0.1% of total protein) to the sample prior to separation, the elution pattern was completely different. Between peaks 1/2 and 3, several broad peaks appeared. In addition, the heights of the other peaks decreased considerably and the resolution was incomplete. Only one band, representing phopholipase A_2, was remarkably resistant to protease degradation (data not shown).

The recovery of the enzymic activity in the elu-

TABLE II

DETERMINATION OF MOLECULAR MASSES AND ISOELECTRIC POINTS OF THE ELUTED PROTEINS

Pancreatic secretory proteins resolved by cation-exchange chromatography were recovered and separated by SDS gel electrophoresis and ultra-thin-layer IEF. Molecular masses and isoelectric points of the proteins were determined by comparison with commercially available marker proteins. For abbreviations and peak numbers see the legend to Fig. 1.

Peak	(Pro)Enzyme	M_r ($\times 10^3$) (this study)	M_r ($\times 10^3$) (from ref. 4)	IEF (this study)	IEF (from ref. 4)
1/2	cbA	47–52	47–52	4.3–5.3	4.3–4.6
1/2	try(a)	25	23.5	4.3–5.2	4.3–4.4
3 + 4	amy 1 + 2	50	53–55	8.5–9.0	8.6–8.9
5	try(b)	25	23.5	8.5	8.0
6	cbB	50	47–52	4.5–5.1	4.3–4.6
8	chy(b)	25	25	9.0–9.3	9.0
10	ela	26	26	9.0	9.2
11	lip	50	50	6.5	6.8

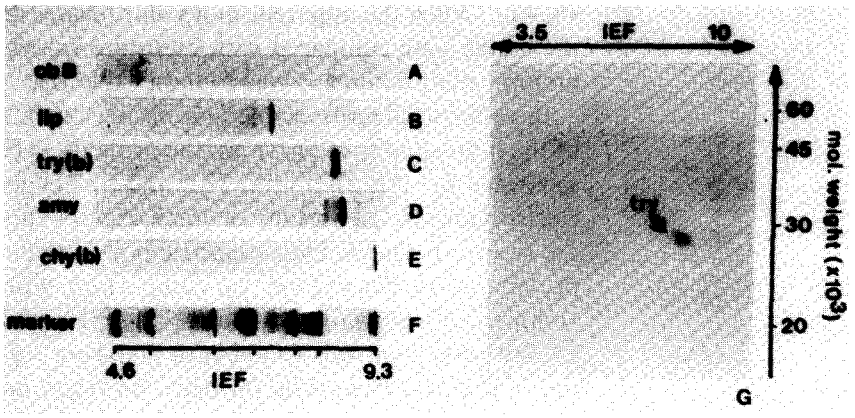

Fig. 4. Separation of eluted proteins by ultra-thin-layer IEF and 2D gel electrophoresis. Column eluates were desalted with NP-5 columns and analysed using IEF (A–F) and 2D gel electrophoresis (G). For abbreviations see Fig. 1. Marker proteins (lane F) were: trypsin inhibitor (isoelectric point 4.6), β-lactoglobulin (5.1), carbonic anhydrase II (5.9), carbonic anhydrase I (6.6), myoglobin (6.8–7.2), lactic dehydrogenase (8.6), trypsinogen (9.3).

ate was near 75% for all enzymes (trypsin 72.2%, chymotrypsin 75.6%, amylase 74.5). To study the reproducibility of this separation technique, one sample of pancreatic juice (320 μg of protein) was run eight times and a different sample (1.6 mg of protein) was used four times. The retention volumes of the peak maxima (measured in millilitres from the onset of separation) show variations below 1% of the mean and are independent of the amount of protein applied (Table III). The areas under the respective peaks were measured by integration. From one run to another, the absolute values varied by *ca.* 5% when 1.6 mg of protein were applied to the column. This value was higher when only 320 μg of protein were used (Table IV).

Radiolabeled proteins were separated by FPLC, and the eluate was collected at 20-s intervals and analysed by β-scintillation counting. No important differences in the UV absorption spectra were observed (data not shown). The re-

TABLE III

REPRODUCIBILITY OF RETENTION VOLUMES AFTER SEPARATION OF PANCREATIC SECRETORY PROTEINS

Pancreatic proteins were analysed by cation-exchange chromatography. Samples with 320 μg (1.6 mg) protein were analysed eight (four) times. Values are mean ± S.D. For abbreviations see the legend to Fig. 1.

Peak	(Pro)Enzyme	V_R (ml)	
		320 μg protein	1.6 mg protein
3	amy 1	6.06 ± 0.17	6.06 ± 0.25
8	chy(b)	11.88 ± 0.22	11.38 ± 0.18
11	lip	14.95 ± 0.15	14.78 ± 0.10
13	plA_2	17.43 ± 0.11	17.51 ± 0.11

TABLE IV

REPRODUCIBILITY OF PEAK AREA AFTER SEPARATION OF PANCREATIC PROTEINS

Pancreatic proteins from rats were separated by cation-exchange chromatography. Two different samples were used. One sample (320 μg) was analysed eight times and another sample (1.6 mg) was run four times. Means ± S.D. of the peak areas were determined. For abbreviations see the legend to Fig. 1.

Peak	(Pro)Enzyme	Peak area (in relative %)	
		320 μg protein	1.6 mg protein
1/2	try(a)/cbA	16.3 ± 1.3	18.5 ± 0.8
3	amy 1	7.7 ± 0.7	8.7 ± 0.8
4	amy 2	6.0 ± 0.9	6.5 ± 0.4
5	try(b)	6.0 ± 0.6	6.7 ± 0.2
6	cbB	7.1 ± 0.8	6.5 ± 0.4
8	chy(b)	4.1 ± 0.4	3.5 ± 0.2
9	chy(a)	24.2 ± 1.0	19.3 ± 1.0
11	lip	9.7 ± 0.9	12.6 ± 0.5
13	plA	18.2 ± 1.7	17.0 ± 0.6
15	RNAse	0.7 ± 0.1	0.8 ± 0.1

TABLE V

PROTEIN PATTERN IN PANCREATIC JUICE AFTER ADAPTATION TO DIFFERENT DIETS

Rats were fed for 10 days with a carbohydrate-poor, fat-rich or protein-rich diet. Radiolabeled methionine was injected and zymogen granules were prepared. The proteins were separated by cation-exchange chromatography, and the UV absorption spectrum and the radioactivity in each protein fraction were determined. The proenzyme composition was expressed as the percentage of total radioactivity and as the percentage of total UV absorption. The values are means of four experiments. The S.D. was below 20% of the mean in each group.

Diet	Radioactivity (% of total)					UV absorption (% of total)				
	amy 1	amy 2	try(a)	lip	chy(a)	amy 1	amy 2	try(a)	lip	chy(a)
Carbohydrate-poor	6.1	2.7	19.3	7.8	19.3	8.2	5.1	22.3	7.1	14.1
Fat-rich	11.8	6.3	17.3	10.6	14.3	15.1	9.1	21.9	8.4	12.4
Protein-rich	14.6	17.5	19.7	5.7	17.6	17.7	10.1	26.2	4.1	13.2

covery of radioactivity was 99.5%. Therefore, determination of pattern of radiolabeled as well as unlabeled enzymes may be possible. To demonstrate this, from rats fed with different diets (carbohydrate-poor, fat-rich and protein-rich diet) zymogen granule proteins labeled *in vivo* were separated by FPLC, and the UV absorption spectra as well as the spectra of radioactivity were analysed. Determination of the respective peak areas showed that the amount of amylase was low when the carbohydrate-poor diet was fed. (Table V). Low fat levels led to a reduced amount of lipase in the pancreatic juice. Similar values were obtained when the pattern of radiolabeled protein was analysed by β-scintillation counting (Table V). In a further analysis we found a high correlation coefficient (r) between the amount of radioactivity of each protein and the respective peak area. Values were $r = 0.92$ for amylase 1, $r = 0.93$ for amylase 2, $r = 0.85$ for basic trypsinogen, and $r = 0.87$ for lipase.

DISCUSSION

This technique for the separation of proteins seems to have several advantages over previously reported procedures. The most intriguing property is the quality of resolution not achieved with any other chromatographic technique, either reversed-phase HPLC or hydrophobic interaction chromatography [11–14, 20]. After a run of less than 40 min, fourteen well-separated peaks were detected. In addition, several minor peaks appeared irregularly. The isoforms of the main enzymes, amylase, trypsinogen, and chymotrypsinogen could be resolved. Procarboxypeptidases A and B were completely separated from each other, which is a difficult accomplishment with high-resolution 2D gel electrophoresis [4].

During the chromatographic run no autoactivation of pancreatic proenzymes occurred, although the methods used were suitable for the detection of minor amounts (less than 0.1%) of active enzymes. Addition of trypsin inhibitors to the buffers is, therefore, not necessary. In contrast to reversed-phase HPLC [12,14] enzymic activity is not destroyed during the separation procedure. The isoelectric points of the proteins determined in the column eluates were comparable with the literature data (Table II).This confirms that the integrity of proteins is maintained during the separation procedure. In accord with this, the recovery of enzymic activity was high. Therefore, in contrast to reversed-phase HPLC, this technique may well be useful for the purification of functionally intact pancreatic secretory proteins.

The elution pattern was destroyed, however, when pancreatic proenzymes were activated prior to separation by addition of small amounts of trypsin. In particular, several early-eluting protein peaks appeared, which were not present in normal pancreatic juice. Therefore, with this

technique, activated and non-activated pancreatic juice can be distinguished. Only phospholipase A_2 was found to be resistant to tryptic hydrolysis. The activity of this enzyme is also preserved during reversed-phase HPLC in the presence of trifluoroacetic acid as solvent [12,14].

The technique described here is very reproducible, with respect to both retention volumes and peak areas, so quantitative determination of the isoenzyme pattern is possible. When a computer-assisted integration is used, the precision of integration may even be higher than found here. Should the peak areas be quantified as done here, an acceptable reproducibility was achieved with a protein amount of 1.6 mg per run. This amount is usually available when pancreatic secretion is studied in conscious rats, because the mean protein concentration in the juice is near 25 mg/ml [7]. In consequence, less than 100 μl of pancreatic juice are necessary. When the zymogen granule content was analysed, in one preparation from a 350-g animal *ca.* 3–4 mg protein were obtained, so that several separations may be performed. If the protein concentration is lower, higher application volumes may be used: with volumes up to 2.5 ml no significant alteration of the separation pattern was detected. Therefore buffer exchange of the sample (*i.e.* by filtration on G10) is not necessary. Only a five-fold excess of buffer A has to be added to the sample and the mixture must be passed through a 0.22-μm mesh. This procedure is rapid and easy and avoids the activation of pancreatic proenzymes, which is regularly observed after filtration on Sephadex [21].

It is remarkable that there was no clear relationship between the isoelectric points of the proteins and the respective elution volumes. From the theoretical point of view using the "net charge" model, acidic enzymes should elute in front of basic forms. This, however, is not the case, as basic proteins such as amylase 1 and 2 (peaks 3 and 4) elute in front of several acidic proenzymes. Similar observations have been made by Kopaciewicz *et al.* [22] in retention-mapping studies on strong ion-exchange columns. These authors were unable to find a strong correlation between isoelectric point and elution volume in more than 75% of the proteins tested. This observation was explained by intramolecular charge asymmetry, which promotes differences in electrical potential on the surface of the protein and allows ionic interaction even when the net charge of the molecule is zero (at the isoelectric point). This may also explain why a complete separation of pancreatic secretory proteins was achieved with a relatively "flat" salt and pH gradient.

When rats were fed with different diets, a characteristic alteration in the pattern of enzymes was found [4]. A high content of starch in the diet increased the percentage of amylase in the juice. Corresponding alterations were found after feeding a high-protein diet or a high-fat diet [4]. In difference to the various one- or two-dimensional gel techniques used to determine isoenzyme patterns [4,5,7,8–10] after separation by FPLC, only the integration of the UV pattern has to be done. Even radiolabeled proteins may be analysed because the spectrum of radioactivity is similar to the UV absorption. After minor modifications the technique could also be used for preparative runs. The separation time may even be shorter, as proteins that are not of interest may be eluted much faster from the column.

In summary, the technique described here may be helpful to approach various separation problems. The procedure is as fast as HPLC and the resolution is much better. Proenzymes were not activated during the run and the enzyme activity is well preserved. The pattern of radiolabeled as well as unlabeled proteins may be determined. The separation method combines the advantages of HPLC and 2D gel electrophoresis and represents, therefore, progress in the analysis of the pattern of pancreatic secretory proteins.

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