Journal of Chromatography, 481 (1989) 233–243
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 669

THE SEPARATION OF PANCREATIC PROCARBOXYPEPTIDASES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND CHROMATOFOCUSING

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(First received January 23rd, 1989; revised manuscript received June 7th, 1989)

SUMMARY

Different experimental conditions and chromatographic supports have been selected for the most efficient and rapid purification of procarboxypeptidases from porcine and human pancreas by different high-performance liquid chromatography (HPLC) variants (anion exchange, reversed phase and gel filtration). Anion-exchange chromatography was found to be the most capable and permitted the isolation, in a single step, of three different porcine procarboxypeptidases (2A + 1B forms) and five different human procarboxypeptidases (2B + 3A forms) in a native and pure state from whole pancreas extracts. Other pancreatic proproteases are also cleanly isolated in the same step. Reversed-phase chromatography under mild conditions separated porcine or human procarboxypeptidases A from other pancreatic proteins in a very short time but was unable further to subfractionate the same proteins. The sequential use of gel filtration (or anion-exchange) and reversed-phase HPLC chromatography permitted, in a simple way, the isolation and dissociation of the strongly bound components of the binary complexes between procarboxypeptidases A and proproteinase E in either porcine or human pancreas extracts. Chromatofocusing on a fast protein liquid chromatographic support was also found to be a very efficient technique, showing a slightly lower capability to separate procarboxypeptidases than anion-exchange HPLC though in a much shorter time and in larger quantities.

INTRODUCTION

The fractionation of pancreatic proteins requires the use of rapid and mild methodologies in order to avoid their cross- and auto-degradation due to their great proteolytic potential and easy activation[1]. In certain species such as the human, only the use of electrophoresis and electrofocusing in the presence of inhibitors and denaturing agents allowed the analytical separation of the different pancreatic proteins in their undegraded forms[2–4]. Making use of this method, Scheele *et al.*[2–5] were the first to succeed in a reliable visualization and quantification of a large number of these proteins.

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However, the above electrophoretic methods are not appropriate for the isolation of these proteins at the preparative level and in a native state. This is particularly true for procarboxypeptidases due to the similar physico-chemical properties of their subspecies[2,3,5], their existence in complexes with other proproteinases or related proteins[6] and their low degree of renaturation once they have been denatured[7]. High-performance liquid chromatography (HPLC) may be particularly well suited to this task given its usual rapidity of operation and high separative powers. Taking advantage of this, several methods have been reported for the isolation of pancreatic hydrolases by HPLC[8–10]. In the case of procarboxypeptidases, a reversed-phase method which allows the isolation of their subfractions together with other pancreatic proteins has also been reported for porcine extracts[11]. Unfortunately, in this case the proteins isolated are denatured since the separation is carried out at low pH.

The present work describes the application of different HPLC variants (anion exchange, reversed phase, gel filtration) to the preparative separation of porcine and human procarboxypeptidases in their native states. The different properties of these variants permit their alternative use in the simple separation of all or of selected procarboxypeptidase subfractions and other pancreatic proproteases. Of particular interest is the isolation of a previously unreported binary complex between human procarboxypeptidase A and proproteinase E. The great potential of the procedures proposed in the general fractionation of pancreatic proteins is also examined. Finally, a procedure for the fractionation of these proteins based on chromatofocusing on fast protein liquid chromatography (FPLC) support is described and compared with the above-mentioned HPLC procedures.

MATERIALS AND METHODS

Pancreatic extracts

Porcine and human pancreas were defatted by treatment with acetone and diethyl ether as described by Folk and Schirmer[12]. Aqueous extracts of pancreatic powders were made in 20 m*M* Tris–HCl (pH 8.0), containing 50 μM bovine pancreatic trypsin inhibitor, for 1 h, at 4°C. Extracts were either precipitated or not with 43% (w/v) ammonium sulphate prior to their chromatographic analysis. In the former case, they were subsequently dialysed against 20 m*M* Tris–HCl (pH 8.0) overnight.

Enzymatic measurements

The free and potential proteolytic activities were measured spectrophotometrically against synthetic substrates: benzoylglycyl-L-arginine (BGA) for carboxypeptidase B[13]; benzoylglycyl-L-phenylalanine (BGP)[12] or furylacryloyl-L-phenylalanyl-L-phenylalanine (FAPP)[14] for carboxypeptidase A; succinyltrialanine-*p*-nitroanilide Suc-(Ala)3-pNA[15] for proteinase E; benzoylarginine ethyl ester (BAEE)[16] for trypsin; acetyltyrosine ethyl ester (ATEE)[16] for chymotrypsin and elastine-congo red for elastase[17]. To measure the potential activities of the respective zymogens, these were treated for 30 min at 37°C with porcine enterokinase (Sigma E-1256) in the case of trypsinogens, and with porcine trypsin (Sigma T-0134) for the analysis of other proproteases, at a zymogen/protease ratio of 10/1 (w/w) in both cases.

Anion-exchange chromatography

A TSK-DEAE column (particle size 10 μm, 100-nm pore, 7.5 cm × 0.75 cm, from LKB) with a guard column packed with the same support was used throughout. The buffers used were: A, 20 m*M* Tris–HCl (pH 8.0); B, 20 m*M* Tris–HCl, 0.8 *M* ammonium acetate (pH 8.0). Elution was achieved by two different gradients: from 19 to 57% B in 110 min for porcine extracts and from 0 to 40% B in 160 min for human extracts. Chromatography was carried out at a flow-rate of 0.5 ml/min, at 20°C.

Reversed-phase chromatography

An Ultrapore C_3 column (particle size 5 μm, 30-nm pore, 10 cm × 0.45 cm, from Beckman) was used, protected by a guard column packed with Perisorb C_2 from Merck. The two elution systems used were: (a) water–acetonitrile, both containing 0.1% trifluoroacetic acid (TFA); (b) water–isopropanol, both containing 10 m*M* piperazine adjusted to pH 6.5 with TFA. Chromatography was carried out at a flow-rate of 0.5 ml/min, at 20°C.

Gel filtration chromatography

A TSK G3000SW gel filtration column (particle size 10 μm, 100-nm pore, 30 cm × 0.75 cm, from Beckman) protected with a Spherogel-TSK GPWH guard column (particle size 10 μm, 7.5 cm × 0.75 cm) was used. Chromatography was carried out in 25 m*M* Tris–HCl, 0.2 *M* NaCl (pH 6.8) at a flow-rate of 0.1 ml/min and 20°C. The column was calibrated with glycogen phosphorylase, bovine serum albumin, chicken egg albumin, carbonic anhydrase, myoglobin and bovine pancreatic trypsin inhibitor.

Chromatofocusing

Chromatofocusing was performed on either a 20 cm × 0.5 cm or in a 5 cm × 0.5 cm Mono P FPLC column (from Pharmacia). The equilibration buffer was 25 m*M* Bis-Tris–HCl (pH 7.1). The pH gradient was achieved by using 10% Polybuffer 74–HCl (Pharmacia) (pH 4.0) as the eluting agent. Under these conditions, a linear gradient between pH 7.0 and 4.0 was formed in about 40 min, at a flow-rate of 0.5 ml/min. Alternatively, and with the longer column, a gradient between pH 5.5 and 4.0 was achieved by using 25 m*M* piperazine (pH 5.8) as the equilibration buffer. The temperature was kept at 20°C. The system was calibrated with standard proteins of known isoelectric points.

Electrophoresis

Electrophoretic analyses were carried out in slab gels of either 12 or 15% polyacrylamide, following Laemmli's method[18], either in the presence of sodium dodecyl sulphate (SDS) (denaturing conditions) or in its absence (non-denaturing conditions).

RESULTS

Anion-exchange HPLC

When an aqueous extract of porcine or human pancreas was made 20 m*M* in Tris–HCl, adjusted to pH 8.0, and subjected to chromatography in a DEAE-TSK

column, a complete separation of the different procarboxypeptidases was observed when they were eluted with a gradient from 0 to 0.32 *M* ammonium acetate. As shown in Figs. 1a and b, two porcine procarboxypeptidases A, the monomer and the binary complex with proproteinase E, and one procarboxypeptidase B were separated in the case of porcine extracts, and three procarboxypeptidases A, two monomers (A2, A1) and the binary complex with proproteinase E (A3), and two procarboxypeptidases B (B2, B1) were separated in the case of human extracts.

The number of the separated subfractions and their molecular properties (see Table I) coincide with those previously observed by our group[1,19] and by others[3–5,20], using different methods. Thus, in the case of the porcine proenzymes, two procarboxypeptidases A, the monomer and the binary complex with proprotei-

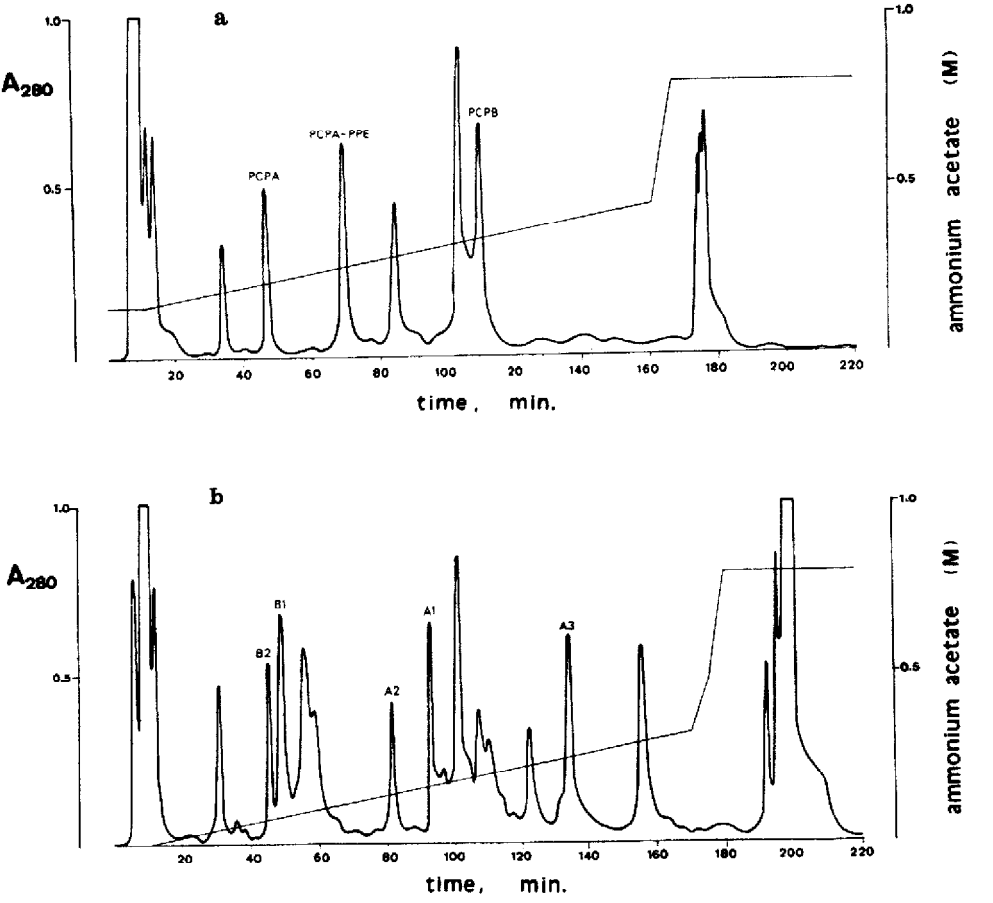

Fig. 1. Chromatographic fractionation of pancreatic proteins in an anion-exchange HPLC column, TSK-DEAE type (7.5 cm × 0.75 cm). Pancreatic extracts were made from 0.2 g of defatted acetone powder: (a) porcine pancreatic extract; (b) human pancreatic extract. Elution was carried out with gradients of ammonium acetate, in the presence of 20 m*M* Tris–acetate buffer (pH 8.0). Flow-rate 0.5 ml/min, temperature 20°C. Assignment of pancreatic procarboxypeptidases was made on the basis of the electrophoretic behaviour of the different fractions and enzymatic measurements, and in agreement with previous reports[2,4,19,20]. See Table I for abbreviations.

nase E, and one procarboxypeptidase B show molecular properties similar to those isolated by Kobayashi *et al.*[20] and by our own group[1,19] using conventional and complex chromatographic methods. In the case of the human proenzymes, procarboxypeptidases B2, B1, A2 and A1 have molecular properties coincident with the same proenzymes as detected and numbered by Scheele *et al.*[3–5] using their two-dimensional electrophoretic method. In addition, a new subfraction, the A3, which is composed of a binary complex between procarboxypeptidase A and proproteinase E from human pancreas is described for the first time. The nature of these proteins was ascertained by electrophoresis in polyacrylamide gels in the presence and absence of SDS determination of their isoelectric points by electrofocusing, analysis of their behaviour in gel filtration HPLC (see below) and by measurement of their actual and potential activity against several synthetic substrates (see Methods). Some of these properties are listed in Table I.

The existence of the binary complex of human procarboxypeptidase A and proproteinase E was demonstrated by its behaviour as a single entity in non-denaturing separative methodologies (electrophoresis, electrofocusing, anion exchange and gel filtration HPLC) and its splitting into two entities when subjected to separative methodologies under denaturing conditions (electrophoresis in SDS or urea, two dimensional electrophoresis in urea gradients, reversed-phase HPLC) (not shown). The identification of proproteinase E as the protein accompanying procarboxypeptidase A was made on the basis of the agreement with properties previously reported for this protein[21–24] and those found for that complex, such as the detection of its activity against Suc-(Ala)3-pNA and the lack of activity against elastin after being treated with trypsin, and the analysis of its N-terminal sequence.

TABLE I

SOME MOLECULAR PROPERTIES OF ZYMOGENS FROM PORCINE AND HUMAN PANCREAS SEPARATED IN THIS WORK BY VARIOUS HPLC TECHNIQUES

The active forms were generated by treatment of the isolated fractions with 0.75 mg/ml of porcine trypsin for 30 min, or with 0.5 mg/ml of enteropeptidase (in the case of trypsinogens) for 30 min, at 25°C. Abbreviations: IEP = isoelectric point; N.D. = not determined; PCPA = monomeric procarboxypeptidase A; PCPA–PPE = binary complex of procarboxypeptidase A and proproteinase E; PCPB = procarboxypeptidase B; CHTn = chymotrypsinogen; Tn = trypsinogen.

Species	*Protein*	M_r, *SDS-PAGE*	*Apparent* M_r, *gel filtration*	*IEP*	M_r *active form, SDS-PAGE*	*Molar activity* (s^{-1})
Pig	PCPA	45 000	29 800	4.7	34 800	9.2
	PCPA–PPE	45 000 + 27 000	53 700	4.7	34 800 + 27 000	10.4–1.38
	PCPB	47 000	29 200	4.4	36 000	114
	CHTn	29 000	18 700	4.2	29 000	48
	Tn	26 000	22 600	N.D.	26 000	31
Human	PCPA2	47 000	33 300	5.1	33 300	60
	PCPA1	44 500	30 800	4.9	33 700	118
	PCPA3	44 500 + 33 000	49 600	4.9	33 700 + 33 000	98–1.26
	PCPB2	47 300	28 700	7.1	35 500	64
	PCPB1	47 300	29 400	6.6	35 500	53
	CHTn	30 000	21 000	7.5	30 000	51
	Tn	31 300	19 400	4.7	31 300	10

All the subfractions isolated using the present method show an almost absolute purity as indicated by subsequent analysis by SDS electrophoresis. Only porcine procarboxypeptidase B and the human procarboxypeptidase A–proproteinase E binary complex require a rechromatography under the same conditions when they are obtained at high loadings of the pancreas extract. In addition, all the subfractions show a complete native state as shown by their easy activation with trypsin and by the high specific activities reached after activation, which compare well with those we have previously reported for the porcine proenzymes[1,23]. It is important to note that, in certain cases the human A3 binary complex obtained by this method was contaminated by trypsin from the preceding chromatographic peak. The quick removal of this contaminant by rechromatography was found to be absolutely necessary to avoid autolysis of A3.

Besides procarboxypeptidases, other pancreatic proproteases are also cleanly isolated in the same chromatographic procedure. Thus, porcine chymotrypsinogen C is cleanly eluted after the binary complex of porcine procarboxypeptidase A and proproteinase E (at 84 min, as shown in Fig. 1a). On the other hand, human chymotrypsinogen is eluted immediately after human procarboxypeptidase B1 and two human anionic trypsinogen are eluted in the peaks before and after human procarboxypeptidase A3 (at 122 and 157 min, respectively, as shown in Fig. 1b). Many other pancreatic proteins can also be purified by slightly changing the elution conditions (not shown). It is also important to note that the above separation of procarboxypeptidases and accompanying proteins can benefit from an improvement in resolution and safety when the pancreas extract is previously fractionated by a salting out precipitation with 43% ammonium sulphate, which removes an important fraction of chymotrypsinogens and trypsinogens from the medium. The supernatant, after an overnight dialysis against 20 m*M* Tris–HCl (pH 8.0), is loaded onto the HPLC column. In this case, the addition of 50 μM bovine trypsin inhibitor to the supernatant to avoid degradation during the long dialysis is advisable.

Reversed-phase and gel filtration HPLC

When a porcine or a human pancreas extract was loaded onto a reversed-phase propyl-bonded HPLC-based column (30-nm pore), equilibrated with 10 m*M* piperazine–HCl (pH 6.5), the pancreatic proteins were eluted in three sharp peaks after application of a linear gradient of increasing isopropanol concentration (0–50%) (results not shown). The last peak contained only a mixture of procarboxypeptidases A, while the penultimate peak contained proproteinase E and other serine proproteinases. The yield of recovery of porcine and human procarboxypeptidases A using this method was found to be inversely dependent on the residence time of these proteins in the column. Thus, after application of a 0–50% isopropanol gradient in 15 min, a 95% recovery of these proteins was noted but the yield decreased to 20% when the same gradient was applied in 30 min. The recovery was even poorer when acetonitrile instead of isopropanol was used as the eluting agent.

Under the above conditions at intermediate pH, both porcine and human procarboxypeptidases B require a much higher percentage of organic solvent to be detached from the column, in which they are eluted at low yield. In contrast, when the chromatography is carried out in 0.1% trifluoroacetic acid but at low pH (pH 2), both procarboxypeptidases A and B (either porcine or human) can be separated from each

other and recovered at a yield which is again dependent upon the residence time of the proteins in the column. However, the intrinsic and potential activity of these isolated proteins, after being equilibrated at intermediate pH and freed of organic solvent by dialysis, is very low ranging from 5 to 20%, in agreement with other reports[25]. It is also worth mentioning that the above chromatographic procedure at low pH dissociates the binary complexes between either porcine or human procarboxypeptidases A and proproteinase E, which can be independently recovered.

Gel filtration HPLC on a silica-based column (TSK G3000SW) was found to be able to separate serine proproteases trypsinogens, chymotrypsinogens, proelastases, etc. from procarboxypeptidases when a porcine or an human pancreatic extract was used a a source of these proteins (data not shown). This was accomplished in spite of an anomalous behaviour of all these proteins which showed a delayed elution and, therefore, an apparent molecular weight lower than expected (see Table I). This behaviour, which can be attributed to non-specific interactions with the support, was not prevented by the use of intermediate ionic strength in the eluting buffer (25 m*M* Tris–HCl, 0.2 *M* NaCl). It was also evident from enzymatic measurements along the chromatographic profiles that in both porcine and human species proproteinase E was present only as a binary complex with procarboxypeptidase A, under a wide range of chromatographic conditions. Unfortunately, other pancreatic proteins of higher molecular weights, such as amylases or lypases were also coeluted with these complexes under the same conditions.

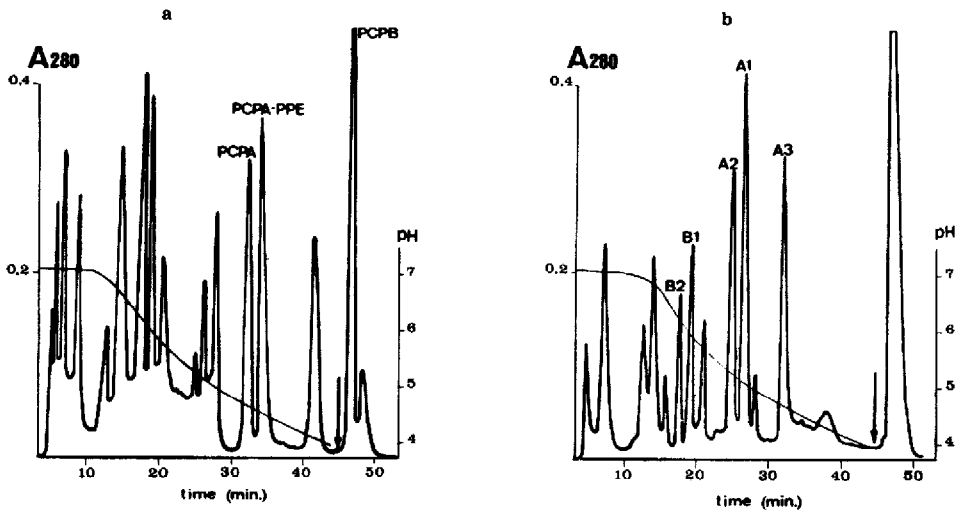

Fig. 2. Chromatofocusing of proteins from a porcine (a), or an human (b), pancreatic extract (each from 16 mg of acetone powder) on a Mono P FPLC column (20 cm × 0.5 cm). Samples were equilibrated against 25 m*M* Bis-Tris (pH 7.1) buffer by dialysis, loaded onto the column and eluted by application of 10% Polybuffer 74–HCl (pH 4.0). Flow-rate 0.5 ml/min, temperature 20°C. Elution of the different procarboxypeptidases and proproteinase E, specifically labelled on the chromatogram, was detected by enzymatic and electrophoretic methods. Arrows on the chromatogram indicate the application of 1.5 *M* sodium acetate (pH 7.1). See Table I for abbreviations.

Chromatofocusing

Chromatofocusing of extracts of porcine or human pancreas in a short FPLC column (Mono P, 5 cm × 0.5 cm, from Pharmacia) gave a good, but not complete, separation of pancreatic proteins when a decreasing gradient between pH 7.0 and 4.0 was applied. However, a complete separation for all these proteins was obtained in a long column (20 cm × 0.5 cm). Thus, as shown in Fig. 2, monomeric procarboxypeptidase A, the procarboxypeptidase A binary complex with proproteinase E and the single procarboxypeptidase B from porcine pancreas extracts are cleanly isolated from each other and from the rest of pancreatic proteins at the end of the chromatography.

A fractionation can also be accomplished for the corresponding proteins from human pancreas but in a more grouped way due to the larger number of isoforms found in this species: a separate doublet of peaks for B2 and B1 is eluted at the first third of the chromatography, a second separate doublet of peaks for A2 and A1 at the middle and A3 is eluted as a single peak before column washing with a high concentration of salt. Enzymatic measurements, polyacrylamide gel electrophoresis (PAGE) and HPLC anion-exchange analysis confirmed the above assignments. It is interesting that the elution order of the A and B forms of procarboxypeptidases is reversed in both species and is in agreement with the important changes observed in the isoelectric points of the B forms (see table I). It is also worth mentioning that a better separation for human procarboxypeptidases A2 and A1 can be accomplished if a restricted gradient between pH 5.8 and 4.0 is applied. However, under these conditions procarboxypeptidases B2 and B1 are eluted together and contaminated with other pancreatic proteins.

DISCUSSION

A systematic survey has been made to study the ability of different new generation chromatographic supports to separate pancreatic procarboxypeptidases. HPLC anion-exchange chromatography on an organic polymeric support has been found to be a particularly appropriate system for separating all subfractions of the above proteins from porcine and human species. Its great separative power, speed and mildness of operation suggest that this system may become a standard method to isolate the above proteins and many other pancreatic proproteases, just as two-dimensional electrophoresis has become at the analytical level[2–5]. The anion-exchange HPLC method is particularly appropriate for isolating pancreatic proproteases, in a native state, at a 100–500 μg level in analytical columns (5 cm × 0.7 cm). This should be sufficient for the characterization of many properties of these proteins by modern procedures. The complementary use of automatic programmers for elution and of autosamplers permits the extension of this separative capability at the milligram level or the consecutive analysis of many samples of different origins. The latter possibility may be very useful in the analysis of the alellomorphism or pathological variants in pancreatic zymogens from different individuals. Moreover, the use of on-line high sensitivity UV detectors allows the detection of pancreatic proteins at an analytical level (≈ 5 μg) which is similar to that reached by two-dimensional electrophoretic techniques with coomassie blue staining.

Other chromatographic variants tested in this work showed a much lower sep-

arative power than anion-exchange HPLC but are still very useful as complementary techniques. Thus, reversed-phase HPLC on a short alkyl-bonded support is unable to fractionate the different species of procarboxypeptidases A. However, this support permits the rapid dissociation and isolation of the components of the binary complex between procarboxypeptidase A and proproteinase E, either from porcine or from human pancreas. This is an absolute requirement for the independent characterization of these components and led in the past to elaborate methodologies for performing similar tasks[6,20,23]. Besides, reversed-phase HPLC is a convenient and very rapid method of concentrating procarboxypeptidases A or eliminating their accompanying salts or low-molecular-weight contaminants. The last application is very convenient for extended N-terminal sequence analysis of procarboxypeptidases, usually contaminated by amino acids and peptides.

Gel-filtration HPLC showed a low general ability to separate pancreatic proteins from porcine or human extracts. Nevertheless, this technique gives useful information about the interaction between pancreatic proteins. Thus, the stabilities of the procarboxypeptidase A–proproteinase E binary complexes over a wide range of ionic strengths and the existence of the human complex have been demonstrated by this technique. Moreover, this technique can be quickly applied to an extract from human pancreas before anion exchange or chromatofocusing in order to remove serine proproteinases from the procarboxypeptidase A–proproteinase E binary complex. This procedure decreases very much the possibility of autolysis of this labile complex whilst facilitating its ionic equilibration before the next chromatography.

The anomalous hydrodynamic behaviour noted for different pancreatic proteins in the HPLC gel-filtration columns used, a fact already reported by other authors and by ourselves with related chromatographic supports[19,26–28], did not seriously affect the studies on the molecular state of these proteins. The results indicate that all the zymogens of porcine and human pancreatic proteases investigated behave as monomers under the elution conditions selected, except those included in the binary complex between procarboxypeptidase A and proproteinase E. Even more important is the observation that proproteinase E activities appear associated only with the peak containing its binary complex with procarboxypeptidase A, a fact also obseved by means of anion-exchange chromatography. This suggests that proproteinase E is not secreted as a monomer. Probably the expression of its gene is coordinated with that of a procarboxypeptidase A, or the latter protein is expressed in excess with respect to proproteinase E and always maintains this protein in a binary complex after binding to it.

Chromatofocusing on an FPLC support also appears an efficient technique for separating pancreatic procarboxypeptidases. The resolution obtained with the long column is lower than that obtained with anion-exchange HPLC but is complete for all porcine or human procarboxypeptidases. In addition, the resolution for human procarboxypeptidases A is greatly increased by application of a narrow pH gradient. Chromatofocusing has the additional advantage of showing shorter elution times, very sharp peaks and a much larger loading capacity which permits the isolation of milligrams of procarboxypeptidases in half an hour. It also offers the advantage over anion-exchange HPLC of a cleaner separation of anionic trypsin (a possible dangerous contaminant at high loadings) from human procarboxypeptidase A3. However, this method suffers from the disadvantage of the need to remove, (*i.e.*, by gel fil-

tration) the Polybuffer for some applications, such as microsequencing or detailed functional analysis.

The isolation of the native binary complex between procarboxypeptidase A and proproteinase E from human extracts exemplifies the efficiency and mildness of the separative methods reported here. Scheele *et al.*[3–5] did not visualize this binary complex using two-dimensional electrophoresis since this was carried out under denaturing conditions which probably dissociated the complex. Even the proproteinase E protomer is not seen by this electrophoretic method, probably because it suffers from autodegradation in the presence of SDS, as suggested by Sziegoleit[29], or when subjected to unfolding conditions prior or during the analysis. The addition of inhibitors of serine proteinases or high concentrations of urea, recommended procedures in Scheele's method[3–5], do not prevent auto-degradations of this protein as we have shown in our laboratory[30].

The demonstration of the existence of a binary complex between procarboxypeptidase A and proproteinase E in human pancreas suggests some ideas about the future research on the state of proproteinases in the pancreas of mammals. According to previous reports[6], procarboxypeptidases occur in non-ruminants mainly as monomers but also as binary complexes with chymotrypsinogen C, as shown in whale[3], or with proproteinase E, as shown in pig[19,20] and now in humans. However, the occurrence of the last complex may be more frequent if the above difficulties in the separation and detection of proproteinase E also appeared in the analysis of the complement of pancreatic proteins carried out in different non-ruminants, such as guinea pig[2], dog[5], hen[32], etc. This would be in agreement with the reports of Sziegoleit and Linder[33] which claim that proproteinase E-like proteins are present in many mammals. The application of the anion-exchange HPLC or chromatofocusing methods described here to the analysis of pancreatic extracts of these species may answer the above questions.

ACKNOWLEDGEMENTS

This work was supported by grant 0385/84 from the CAICYT, Ministerio de Educación y Ciencia (Spain) and by grant AR/86-14 from the CIRIT, Generalitat de Catalunya (Spain). R. P. is a recipient of a fellowship from Fondo de Investigaciónes Sanitarias (FIS) (Ministerio de Sanidad, Spain), and F. J. B. is a recipient of a fellowship from PFPI (Ministerio de Educación, Spain). We are indebted to Drs. Quintanilla and Espinel from the Unidad de Transplantes, Hospital Valle del Hebrón, Barcelona, for supplying human pancreas.

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